Dissertation

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Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

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Universität Zürich

von

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Zürich, 2009

Diese Arbeit wurde in der Abteilung für Klinische Chemie und Biochemie (Leiter: Prof. Dr. C. W. Heizmann) und der Abteilung für Onkologie (Leiter: Prof. Dr. med. F. K. Niggli) des Kinderspitals Zürich, unter der Anleitung von Dr. A. Arcaro durchgeführt.

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ABBREVIATIONS

Arp	actin related protein
ATP	adenosine triphosphate
BC	breast cancer
cDNA	complementary DNA
CR1/CR2/CR3	conserved region1/2/3
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF-4E	Eukaryotic translation initiation factor 4E
EMT	epiteliomesenchymal transition
ER	estrogen receptor
ERK	Extracellular signal-regulator kinase
Erk1/2	extracellular signal-regulated kinase 1/2
FAK	focal adhesion kinase
FKHR	forkhead in rhabdomyosarcoma
FRAP	FK506 binding protein 12-rapamycin associated protein 1
GAPs	GTPase activating proteins
GEFs	guanine nucleotide exchange factors
grb2	growth factor receptor-bound protein 2
GS domain	glycine and serine domain
IGFR, IGF-IR	insulin-like growth factor receptor, insulin-like growth factor I receptor
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MEK	mitogen-activated Erk kinase
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin-rictor complex
NES	nuclear export signals
NF-kB	nuclear factor-kappa B
PDGFR	platelet-derived growth factor receptor
PDGFR	platelet-derived growth factor receptor
PDK1	phosphoinositide-dependent kinase-1

PDK2	phosphoinositide-dependent kinase-2
PH	pleckstrin homology domain
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
PTB	phosphotyrosine-binding
PtdIns	phosphatidylinositol
PtdIns(3)P	phosphatidylinositol (3)-phosphate
PtdIns(3,4)P_2	phosphatidylinositol (3,4)-bisphosphate
PtdIns(3,4,5)P_3	phosphatidylinositol (3,4,5)-trisphosphate
PtdIns(4,5)P_2	phosphatidylinositol (4,5)-bisphosphate
PTEN	phosphatase and tensin homolog deleted on chromosome 10
pTyr	phosphotyrosine
RTK	receptor tyrosine kinase
S6K	ribosomal protein S6 kinase
SCLC	small cell lung cancer
SH2	Src homology-2
SH3	Src homology-3
shRNA	short hairpin RNA
Smad	small mothers against decapentaplegic
TGF-α/TGFβ	transforming growth factor α/β
VEGF	vascular endothelial growth factor
VEGF	vascular endothelial growth factor
WASP	Wiskott-Aldrich Syndrome protein

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ABBREVIATIONS

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ECM	extracellular matrix
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EGFR	epidermal growth factor receptor
eIF-4E	Eukaryotic translation initiation factor 4E
EMT	epiteliomesenchymal transition
ER	estrogen receptor
ERK	Extracellular signal-regulator kinase
Erk1/2	extracellular signal-regulated kinase 1/2
FAK	focal adhesion kinase
FKHR	forkhead in rhabdomyosarcoma
FRAP	FK506 binding protein 12-rapamycin associated protein 1
GAPs	GTPase activating proteins
GEFs	guanine nucleotide exchange factors
grb2	growth factor receptor-bound protein 2
GS domain	glycine and serine domain
IGFR, IGF-IR	insulin-like growth factor receptor, insulin-like growth factor I receptor
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MEK	mitogen-activated Erk kinase
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin-rictor complex
NES	nuclear export signals
NF-kB	nuclear factor-kappa B
PDGFR	platelet-derived growth factor receptor
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SH2	Src homology-2
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shRNA	short hairpin RNA
Smad	small mothers against decapentaplegic
TGF-α/TGFβ	transforming growth factor α/β
VEGF	vascular endothelial growth factor
VEGF	vascular endothelial growth factor
WASP	Wiskott-Aldrich Syndrome protein

1A SUMMARY

Breast cancer is one of the most prevalent cancers affecting women. When early detected it has a very good prognosis, but once metastasis (cancer cells spreading out from the primary tumor site occurs) occurs, the average survival time decreases drastically.

In recent years, there has been an explosion of life-saving treatment advances against breast cancer. Unfortunately most of our understanding regarding breast tumor stages is quite old, and few new markers for prognosis have been developed in the last few years. One of the most difficult aspects in the treatment of breast cancer in fact, is the absence of specific markers to identify the stage of the tumor, which complicates the design of specific and effective cures.

In the past, most patients were treated in an overly aggressive way (oophorectomy, breast excision, or highly toxic chemotherapy), or in a too mild way resulting in increased mortality because of undetected metastasis.

The elucidation of the mechanisms responsible for the highly invasive potential cancer cells may help identification of specific targets for the diagnosis and treatment of cancer patients. Cell migration is one of the key events in invasive cancer and metastasis. Tumorigenic cells acquire this characteristic by undergoing a phenotypic mutation called epitheliomesenchymal transition (EMT).

Our long-term goal was to elucidate the signal cascade leading to the EMT as a prerequisite to identify new markers specific for different stages of breast cancer.

The specific observations behind this project are that both Ras up-regulation which is frequently found in breast cancer patients, and TGF-β stimulation, are able to transform normal epithelial cells into malignant fibroblastoid cells. The experimental focus of this work, therefore, was to elucidate the signaling pathways involving both, Ras protein and the TGF-β receptor.

PI3K was shown to play a central role in upregulating migration and invasion of cancer cells. PI3K is a family of signaling enzymes subdivided into three classes (I-III) on the basis of sequence homology and substrate preference.

Our experiments revealed that two members of class IA PI3Ks (p110□ and p110□) were able, when over-expressed, to activate downstream signaling proteins such as S6K, cdc42 and Rac and induce migration in cancer cells.

At the same time an opposite role for the third member of the Class IA PI3K, p110□β, was also evident. In fact, p110□ over-expression reduced epithelial cell migration, while its down-regulation, together with some downstream targets, (Rho, Akt), increased migration of breast cancer cells. These contrasting roles for PI3K isoforms, were confirmed by using specific pharmaceutical inhibitors as well as RNAinterference (RNAi).
Together these results demonstrate that specific enzymes are involved in the acquisition of a malignant invasive phenotype of breast cancer cells. We also proved that decreasing p110α, p110β, S6K and cdc42/Rac expression or increasing p110δ, AKT and Rho levels can reduce migration.

In another study, we demonstrated that class II PI3K also plays an important role in cell migration and proliferation. In fact, we observed that PI3KC2β associates with the Eps8/Abi1/Sos1 complex and is recruited to the EGF receptor as part of a multiprotein signal complex.

We also documented an over-expression of PI3KC2β in subsets of tumors and cell lines from acute myeloid leukemia, medulloblastoma and small cell lung cancer (SCLC). Here we could confirm a crucial contribution for PI3KC2β in inducing cell proliferation and survival.

Our recent data then proved that class II PI3KC2β can be tyrosine phosphorylated and activated by Src. In the PI3KC2β protein sequence several tyrosine residues were identified as possible site for phosphorylation by Src. Which residues play an essential role in activating or inhibiting PI3KC2□ kinase activity, is still under investigation.

Together these data regarding PI3K isoforms described new specific pathways to target in order to develop new anticancer therapies, although this would still require further studies.

1B ZUSAMMENFASSUNG

Brustkrebs ist die häufigste Krebsart, von der Frauen in der Welt betroffen werden. Die Früherkennung der Krankheit bietet den Patienten gute Möglichkeiten an, den Tumor zu bekämpfen. Sobald die Zellen sich vom primären Tumor abtrennen, um Metastasen zu bilden, nehmen die Überlebenschancen drastisch ab.

In jüngster Vergangenheit hat es eine Explosion an lebensrettenden Behandlungen gegen den Brustkrebs gegeben. Unglücklicherweise sind die Kenntnisse der Tumorstadien relativ alt und in den letzten Jahren wurden nur wenige neue Markers für Prognosen entwickelt. Eine der schwierigsten Aspekte bei der Behandlung von Brustkrebs ist der Mangel an spezifische Tumormarker um das Stadium des Tumors zu identifizieren. Dies erschwert die Entwicklung spezifischer und somit effektiver Behandlungsmethoden.

In den vergangenen Jahren wurde die Mehrheit der Patienten entweder in einer sehr aggressiven Weise (Oophorectomy, Brustentfernung oder sehr hohe Dosen an Chemotherapie), oder in einer zu sehr milden Weise behandelt. Letztere führte auf Grund der unentdeckten Mestasatse zu zunehmenden Todesfällen. Die Aufklärung der Mechanismen, welche den Krebszellen ihre invasive Eigenschaften verleihen, kann uns helfen, spezifische Zielmoleküle für die Identifizierung und Behandlung von krebskranken Patienten zu finden. Migration ist ein Merkmal bei invasiven Krebszellen und Metastasebildung. Tumorzellen erwerben diese Fähigkeit, in dem sie eine phänotypisch Mutation durchmachen, die Epithelio Mesenchymale Transition (EMT) bezeichnet wird.

Das langfristige Ziel der vorliegenden Arbeit war, die Aufklärung von Signalkaskaden welche Tumorzellen während der EMT führen. Dies ist die Voraussetzung um neue spezifische Markers für die Identifizierung der verschiedenen Stadien des Brustkrebses zu ermitteln.

Die Tatsache, dass sowohl Ras-Protein up-Regulation (Ueberexpression) als auch TGF-β Stimulation in der Lage sind, Epithelzellen in Fibroblasten zu transformieren, wurde als Ausgangspunkt dieser Arbeit festgelegt. Der experimentelle Schwerpunkt dieser Studie lag daher darin, den Signaltransduktionsweg aufzuklären, welche das Ras-Protein und den TGF-β-Rezeptor beteiligt.

Frühere Studien belegen, dass PI3K eine zentrale Rolle bei der upregulating von Migration und Invasion von Krebszellen spielt. PI3K bilden eine Superfamilie von signalisierenden Enzyme, die nach Sequenzhomologie und Substratspezifität in drei funktionellen Klassen (I-III) unterteilt werden.

Unsere Untersuchungen offenbarten, dass zwei Mitglieder der Klasse IA PI3Ks (p110α und p110β) in der Lage sind, nachgeschaltet Signalprotein wie S6K, cdc42 und Rac zu aktivieren und induzieren Migration in den Krebszellen, wenn sie überexpriemiert sind.
Gleichzeitig wurde die gegensätzliche Rolle des dritten Mitglieds der Klasse Iα PI3K (p110δ) nachgewiesen. Die Überexpression von p110δ verringerte Migration, während ihre Hemmung, zusammen mit anderen nachgeschaltet Target wie Rho oder Akt, Migration bei Brustkrebs wieder ansteigen liess.
Diese gegensätzlichen Rollen der PI3K wurden durch verschiedene Untersuchungen mit gezieltem RNAinterference (RNAi) oder mit Hilfe spezifischer Inhibitoren. bestätigt
Alle diese Resultate zusammen haben bewiesen, dass spezifische Proteine, an der Akquisition des bösartig invasiver Phänotyp der Brustkrebszelle beteiligt ist. Des weiteren haben wir bewiesen, dass eine abnehmende expression von p100α, p110β, S6K und cdc42/Rac oder eine Erhöhung der p110δ, AKT und Rho Levels, die Bösartigkeit verringern kann.

In anderen Studien haben wir bewiesen, dass die Klasse II PI3K auch eine wichtige Rolle bei der Migration und Proliferation der Zellen spielt. Tatsächlich haben wir beobachtet, dass sich PI3KC2β mit dem Eps8/Abi1/Sos1-Komplex verbindet und dass der EGF Rezeptor diese rekrutiert als ein Teil eines Multiprotein Signalkomplex.

Neue Daten haben bewiesen, dass Klasse IIβ durch Src phosphoryliert und aktiviert wird. Entlang der PI3KC2β Proteinsequenz wurden mehrere Tyrosine als mögliche Stelle zur Phosphorylisierung durch Src erkannt. Welche Position eine unentbehrliche Rolle bei der Auslösung oder Verminderung der Kineaseaktivität spielt, wird immer noch erforscht.

All diese Daten betreffend PI3K könnten zum Angriff auf einen neuen spezifischen Signalweg führen um neue und präzise Antikrebs-Therapien zu entwickeln, wobei diese Annäherung weitere Studien benötigen würde.

2 INTRODUCTION

2.1 Breast Cancer

Breast cancer remains the most common malignancy among women in the Western world.
It is not a disease of modern society, but has become a major health problem over the last 50 years, affecting as many as one in eight women during their life time. The annual incidence rates in the low-resource countries are increasing up to 5% a year [1]. It is estimated that every year this type of tumor affects more than a million women all around the world, and around 400´000 die from this pathology. The incidence and gravity of this tumor are increasing with the increasing age [2].
In the early stage of development the breast is immature and structurally similar in male and female. In girls the first rapid period of development is puberty, where hormones such as progesterone and estrogen induce the breast tissues to rapidly grow and differentiate more [3]. Histological analysis reveals that the normal breast epithelium is composed of a relatively simple bilayer of inner luminal cells required for milk production and an outer layer of myoepithelial cells required for milk ejection. All the tissues are supplied with blood from a net of axillary, internal mammary and intercostal system of arteries and veins. It also contains several groups of lymph vessels [4]. Both the blood and the lymphatic system serving the breast, are responsible for cancer cell circulation and metastasis formation. More than 95% of breast cancers develop from the epithelial cells, arising from either the milk producing glands (lobular carcinoma) or the draining ducts (ductal carcinoma) [5].
The clinical manifestation of breast cancer can range from a localized tumor to a widely metastatic neoplasm, and is mainly divided into stages depending on size, localization and presence of metastasis [5].

STAGE 0: This stage includes the ductal carcinoma in situ. Here the tumor is still confined in the duct and cancer cells have not invaded surrounding tissues.

STAGE I: The tumor measure at least 2 cm and some of the cancer cells spill out of the duct. From the biopsy there is no evidence of cancer cells in the lymph nodes.

STAGE II: The tumor is increasing in size, and measures around 5 cm maximum but still there is no evidence of cancer cells in the lymph nodes.

STAGE III: Here the cancer has really taken hold in the lymph nodes. The size of the primary mass is not important, but if the lymph nodes are involved, the tumor is anyway classified as stage III [6].

STAGE IV: The tumor has spread out leading to secondary tumors. Tumor cells can disseminate in many ways, locally (to the underlying chest wall and related structures), regionally (via the lymphatic system cancer cells arrive to the axillary, supraclavicular or contralateral lymph nodes), systemically (via the bloodstream to distant sites including bone, liver, lungs, brain and ovaries).

The overall survival rate of patients with early advanced breast cancer (BC) has increased over the years largely because of adjuvant therapy [7-10]. Unfortunately the outlook for metastatic breast cancer remains extremely poor and patients mainly die from metastases rather than uncontrolled local disease. Therefore early detection of the tumor is a potentially important strategy for reduce mortality

In general the treatment options that can be considered are surgery, radiotherapy, cytotoxic chemotherapy and hormonal manipulation to endocrine therapy. The chosen option is in general according to the tumor stage. For example, patients whose lesions are tightly focused can be treated with surgery. The aim of this treatment is to achieve local control of the disease and prevent complications. It can be radical (mastectomy) [11, 12] or conservative (lumpectomy/quadrantectomy) [13-15].

Very often surgical treatment is performed in combination with adjuvant therapy like radiotherapy (ionizing radiation) and chemotherapy (cytotoxic drugs). In general all the systemic therapies given after surgery to patients, who have no evidence of spreading of cancer, are called adjuvant therapy. When used as adjuvant therapy, after breast-conserving surgery or mastectomy, chemotherapy and radiotherapy reduce the risk of breast cancer recurrence [16]. Even in the early stages of the disease, cancer cells can break away from the primary breast tumor and spread through the bloodstream. These cells do not cause any symptoms, they do not show up on imaging tests, and they can not be felt during a physical exam. But if they are allowed to grow, they can establish new tumors in other places in the body. The goal of adjuvant therapy is to kill undetected cancer cells that have traveled from the breast [17]. Chemotherapy is also largely used in advanced breast cancer, before surgical therapies, in order to shrink large cancers so that they are more circumscribed and small enough to be removed [18].

The recognition of the role of estrogens in inducing breast tissues to rapidly grow and differentiate, opened the path to investigate their role in breast tumor development. The evidence of its role in stimulating breast tumor growth was quite immediate and this led to the development of therapies based on endocrine intervention[19].

The predominant source of hormones in premenopausal women are the ovaries. In postmenopausal women they cease to produce them as a result of a complete loss of primordial follicles [20]. However, estrogens continue to be synthesized through the conversion of circulating androgens (namely, androstenedione and testosterone) to estrogens (estrone and estradiol) [21]. Further to these evidences nowaday ovarian suppression is an established treatment for breast cancer in premenopausal women, achieved surgically (oophorectomy) [22], by radiotherapy and recently also medically (using luteinizing-hormone releasing-hormone antagonists such as Zoladex) [23]. This approach is potentially reversible and is associated with less morbidity than radiotherapy [23].

For postmenopausal patients selective estrogen receptor modulators (SERMs) and selective estrogen receptor down regulators (SERDs), which inhibit the proliferation stimulus on breast cancer cells are employed. Tamoxifen is the most investigated and most widely used representative of these agents, and has been introduced in the advanced disease, in the neoadjuvant and adjuvant setting, and for the prevention of the disease [22].

The most abundandt source of estrogens in postmenopausal women are the androgens present in the peripheral tissues. These are transformed in to estrogens by the enzyme complex called aromatase. Inhibition of aromatase is an important approach for reducing estrogen levels in postmenopausal women, thus reducing their effect on breast tumor cell proliferation [24]. In general anyway, endocrine or hormonal therapy has an effect only on those women whose tumors are positive for estrogen receptors (ER) and/or progesterone receptors (PgR). Therefore these receptors are usually measured in every primary breast cancer, as well as in metastatic lesions where the results would influence a treatment decision [25].

Another protein status that is now routinely tested is the HER-2/neu oncogene. Infact it became clinically relevant with the demonstration that HER2-povitive breast cancers are more aggressive and have a worse prognosis that HER2-negative tumors [26]. The HER2/neu proto-oncogene is a member of a family of genes encoding transmembrane receptors including the epidermal growth factor receptor (EGFR) and regulates important aspects of the growth and differentation of cells [27]. Recent new therapies, still under investigation, have focalized their attention on blocking angiogenesis. When the tumor grows in size, it requires

blood supply and for this it releases factors inducing neovascularization [28]. By blocking factors or the specific receptors the growth of the tumor can be put under control. Numerous VEGF-based antiangiogenic therapies are currently being evaluated in phase II and III clinical studies, which should clarify their potential clinical use [29].

The VEGF protein is overexpressed in more than 30% of breast cancers and these tend to be hormone-receptor-negative, high-grade and aggressive tumors. On the other hand, these tumors are most likely to respond favorably to the other therapies [30].

As presented until now, the main prognostic factors that have been used to predict the course of the tumor development and thereby its treatment are patient age, tumor size, status of axillaris lymph nodes and hormone-receptor status.

Because these factors, when used individually or in combination, do not yield prognoses with an extremely high degree of accuracy, the great majority of patients diagnosed with primary breast cancer are treated in an aggressive way even if only few presented at the end metastasis [31, 32]. Many treatments presented until now have very strong and delayed side effects, which are much worse than their advantages in breast cancer treatment. In order to avoid them, gene expression profiling can allow to predict the clinical course of breast cancer progression with higher accuracy [32, 33].

In addition, the outlook of the patients submitted to the current treatment is really poor.

The treatments have been improved much faster in the past than how was done in the last 20 years, where no effective new drugs were efficiently developed. Tumor gene expression profililing and thereby recognition of new targets could be an efficient alternative therapeutic strategy [33].

2.2 TUMOR SIGNAL TRANSDUCTION

2.2.1 Tumorigenesis

Cancer is a group of diseases where a cluster of cells stop to answer to the normal physiological input of cell control as a consequence of DNA damages.

Cancer cells differ from normal cells in many important characteristics common to all tumors:

1- Uncontrolled and unlimited ability to proliferate [34].
2- Absence of contact and density inhibition (normally cells proliferate until they reach a specific density after which they become quiescent) [35].
3- Reduced adhesion capacity, due to the capability to migrate more [36].
4- Angiogenesis, a physiological process involving the growth of new blood vessels from pre-existing vessels [29].
5- Resistence to apoptotic signals [37].
6- Unilimited proliferative potential [38].

In normal cells, replication is a stimulus coming from the environment and able to drive cells in dividing into two by entering into mitosis. Mitosis is a cell process divided in 4 phases (G1/S/G2/M) separated by **checkpoints** that ensure the fidelity of the cell division. These checkpoints verify whether the processes at each phase of the cell cycle have been accurately completed before progression into the next phase. This means that the DNA is checked in each phase [39]. The increased incidence of cancer as a function of age has long been interpreted to suggest that genetic changes are require for tumorigenesis [40].

Very often in fact the checkpoint machinery recognises errors and then the damage response signalling pathway intervenes in repairing them [34]. The first step of this pathway consists in silencing proteins responsible for driving the cell in to the next phase, in order to stop the cell cycle and give more time to the cell for repair the damage. Cells that fail to properly repair DNA damage and that undergo mitotic slippage will be eliminated by committing to the apoptotic program, thus preventing the emergence of cells containing an aberrant and unstable genome. Sometimes the molecules that link the mitotic checkpoint and the apoptotic program fail and the damaged cell goes on proliferating and giving rise to a population of cells carrying mistakes in their genome[39]. This is thought to be the first step of carcinogenesis, therefore when damages are accumulated, the cell gives rise to a tumor. The first evidence of

tumorigenesis is that some cells start to proliferate in an uncontrolled way and without requiring the presence of stimulators in the surrounding. Several proteins involved in proliferation undergo mutations in their gene sequence, coding after that for a constitutively active protein that induces proliferation (oncogene) or for an ineffective inhibitory protein (tumor suppressor gene). The vital clues about oncoprotein functioning came from detailed studies of how normal cells regulate their growth and proliferation [41]. Therefore, to truly understand the complexity of oncoprotein function, we need to understand the details of how normal cell proliferation is governed. Normal cells recognise the presence of growth-stimulatory factors present in the proximity via specific receptors expressed on the surface [42]. The received signal is internalised and integrated by complex circuits within the cell, which decide whether cell growth and division is appropriate or not.

These extracellular signalling molecules can be very small and pass directly through the plasma membrane. Otherwise, as in the case of most of the signalling molecules, they need a receptor to translate their message from the outside to the inner cytoplasmatic cell compartment. All these receptors are composed in general by an extracellular domain that recognises and binds to the stimulatory factor. Then there is a transmembrane domain anchors the protein in the membrane and finally a cytoplasmic domain which is able to interact with downstream proteins, carrying on the signal.

Cell surface receptors can be subdivided int groups depending on the way they transmit the signal. The most important groups are: **integrins, tyrosine kinases**, **serine-threonine kinases and G protein-coupled receptor**.

2.2.2 Tyrosine Kinase Receptors (RTKs)

The study of tyrosine kinase receptors began with the discovery of the epidermal growth factor (EGF), the first of the growth factors to be discovered [43].

The EGFR consists of a number of extracellular domains forming the ligand-binding site [44], a transmembrane sequence and juxtamembrane domain, followed by an intracellular kinase domain and C-terminal domain [45]. This receptor is expressed in all epidermal and stromal cells as well as some glial and smooth muscle cells. It presents the ability to recognise and bind several ligands, including EGF itself, transforming growth factor (TGF-α) [46], and heparin-binding EGF (HB-EGF) [47]. Upon activation by its growth factor ligands, the EGFR undergoes a transition from an inactive monomeric form to an active homodimer even if there are some evidences that preformed inactive dimers may also exist before ligand binding. Since the EGFR has three known homologues, ErbB2 (Neu or HER2), ErbB3 (HER3) and ErbB4 (HER4), in addition to forming homodimers it may pair with another member of the family, to create an activated heterodimer [47]. This dimerization is essential in order to activate the kinase activity of these receptors. In fact only after dimerization these two receptor chains are able to reciprocally phosphorylate the tyrosine kinases present in their long carboxy terminal cytoplasmatic tail [47], inducing conformational change in the cytoplasmatic tail activating the signal [48]. In particular, several tyrosines that can be phosphorylated providing a docking sites for proteins containing SH2 (Src homology region 2) and PTB (phospho-tyrosine binding) domains were identified [49]. The Src homology 2 domain (or SH2 domain) is a protein domain of about 100 amino acids first identified as a conserved sequence region among the oncoproteins Src and able to recognise and bind phosphorylated tyrosine residues [50, 51]. More precisely it also recognises the amino acids present at the sides of the phosphorylated tyrosine making the binding site extremely specific. The evidence that the binding of a particular SH2 domain to tyrosine-phosphorylated proteins is dependent on the primary sequence around the phosphotyrosine (pTyr) came from studies on the PDGF (Platelet-derived growth factor) receptor, that is an other member of the tyrosine kinase receptors, structurally similar to the EGFR [50, 52, 53]. Another important member of this group of receptors is the VEGF (vascular endothelial growth factor) receptor. This receptor was demonstrated to play an essential role in endothelial cells differentiation and, during development, in the vascular system morphogenesis [54]. As previously mentioned, VEGF is also essential in tumors that

need a process of neovascularization able to bring nutrients for expansion and progression [55]. This growth factor binds two high-affinity receptor tyrosine kinases, VEGFR1/flt-1 and VEGFR2/flt [56], both present in normal endothelial cells and upregulated during angiogenesis, despite VEGFR2 mediating most of the angiogenic functions attributed to VEGF [57]. VEGF has been liinked to poor prognosis in breast cancer [58]. Numerous studies have shown a decreas in patient outlook in those tumors overexpressing the VEGF receptor. The overexpression of VEGF may be an early step in the process of metastasis, a step that is involved in the "angiogenic" switch.

Regarding its possible role in cancer, in general all the growth factor receptors can fundamentally be oncoproteins. In fact, since all the growth factor receptors can activate mitogenic signaling pathways, the idea of investigating the role and the sequence of these receptors in tumors was immediate.The first observation was an autocrine induction of these signals [59]. Rather than a growth factor signal sent from one cell to another located nearby (paracrine signaling), or sent through the circulation to distant tissues (endocrine signaling), cancer cells perform an autostimulation also known as autocrine signaling loop, in which cells produce their own mitogens. Tumors make and release growth factors to which they can also respond [60, 61]. Very often cells able to produce their own growth factors (autocrine proliferation induction), overexpress the relative receptor, or if tumor cells do not express it at all, they can even start to produce the mRNA and then the receptor [62-64]. Following dimerization and transphosphorylation models, there were new observations regarding the possible role of growth factor receptors in inducing cancer formation. In fact, since these receptors are free to move in the plasma membrane, their increased numbers can cause them to bind frequently and these events are comparable to the dimerization events caused by ligand binding, triggering phosphorylation, receptor activation and signal emission, despite the absence of the ligand [65]. For instance, the EGFR receptor is overexpressed in a wide variety of human tumors, mostly carcinoma [66]. In such tumors, this can result in ligand-independent receptor dimerization and activation, or alternatively, can make cancer cells hyper-responsive to low levels of EGF and TGF ligands [65]. This original theory was recently replaced by some evidences suggesting the involvment of G protein-coupled receptor (GPCRs) in EGFR activation [67]. In general overexpression of the receptor can induce the signal even without ligand. This overexpression can be due to mutations in the DNA, like amplification that has been demonstrated to occur in different tumor types [68, 69]. This was

present in more than 40% of the total solid tumors analysed. Several other genetic alterations like region deletion (like in the regulatory site of the gene expression), tandem duplication or point mutation were also identified [70, 71].

All these mutations, beyond inducing receptor amplification, can code for a constitutively active form of it, active in a ligand-independent way [72]. Alterations like N-terminal truncation or tandem duplications, small deletion or some point mutation can also be involved. For instance, a point mutation can be present in the sequence coding for the transmembrane domain of the receptor and the resultant mutation could favor the constitutive dimerization of the two receptors chains. Some changes can be due to substitution of the tyrosine present in the catalytic domain with an amino acid able to mimic phospho-tyrosine, like Asp (aspartic acid) and Glu (glutamic acid) [73]. In this way, the receptor gives a continuous signal to downstream proteins for the induction of cell proliferation [74]. After growth factor stimulation, cells acquire the ability to not only proliferate more but also to migrate and to survive longer. This means that the receptor is able to transduce several types of biochemical signals and that these signals impinge on a diverse array of cellular targets [75]. Understanding how signaling cascades operate was a crucial point to comprehend tumorigenesis. In fact, oncoproteins flood cells with a continuous stream of mitogenic signals, thereby the transformed state of cancer cells might well represent an exaggerated version of the responses that normal cells exhibit following exposure to growth factors. The first step following the activation of RTKs is the recruitment of adaptor proteins able to bind to the phosphorylated cytoplasmic domain of the receptor and in turn to induce activation of downstream proteins like Ras. The first adaptor protein identified is Grb2, a 25kD protein composed almost entirely of SH2 and SH3 domains [76]. Its SH2 domain is able to bind the activated cytoplasmatic domains of RTKs. This domain is approximately 100 amino acid-long and was identified in many cellular proteins with the role of binding phosphotyrosine residues present in the target protein. It is able to recognise the phospho-tyrosine and the amino acids surrounding it in a specific way [51, 53]. The Grb2 protein name, standing for growth factor receptor-bound protein 2, reflects the protocol by which this protein was identified, which was by screening a bacterial cDNA expression library with the tyrosine phosphorylated carboxy terminal region of the EGFR [76, 77]. Some adaptor proteines present only this SH2 domain in their sequence, but most of the signalling proteins in general also present other domains responsible for driving on the signal cascade [78]. Frequently together with the SH2 domain an SH3 domain, that commonly recognizes proline-rich sequences, is also present.

This domain was shown from many evidences to be able to interact with guanine nucleotide exchange factors and GTPase-activating proteins which in turn regulate the activity of small G proteins like the Ras superfamily [78-80]. Regarding the Grb2 protein, the SH3 domain turned out to be responsible for the binding to a second adaptor protein (Sos) that acts as a nucleotide exchange factor (GEF) [81]. RTK-Grb2 interaction is then essential for the translocation of Sos to the plasma membrane where it is closer to the Ras protein and can stimulate it to release GDP and bind to GTP, thereby activating it. Mammalian cells contain two closely related counterpart of Drosophila SOS: Sos1 and Sos2 [82]. Their protein product has a molecular mass of ~150 and is composed of several defined domains. Biochemical studies have established that Sos indeed functions as a guanine nucleotide exchange factor for Ras. Its catalytic activity is conferred by a 450-amino-acid domain (CDC25) that is highly conserved among different Ras exchange factors and highly specific for Ras [83]. Sos presents a second SH3 domain able to bind other adaptor proteins like Shc and E3b1 (or Abi-1). These three proteins together form a complex in which they bind each other through SH3 domains [84-86]. The NH_2-terminal region of Sos contains regions of homology with Dbl (DH) and pleckstrin (PH) domains. This structure, the DH domain followed by a PH domain, is a typical motif of guanine nucleotide exchange factors that are specific for Rho GTPases family members (Rho, Rac, CDC42). The DH domain is the catalytic entity mediating the guanine nucleotide exchange on Rho GTPases, whereas the PH domain appears to be involved in the modulation of nucleotide exchange activity through several possible mechanisms, including subcellular targeting and intramolecular interactions. The existence of a DH-PH module in Sos, along with the abundance of evidence linking Ras activation to the activation of Rac, has created a framework for the idea that in addition to its guanine nucleotide exchange activity toward Ras, SOS might be endowed with a catalytic activity that promotes guanine nucleotide exchange on Rac [87]. In support of this idea, overexpression of the DH domain of Sos leads to the activation of Rac by enhancing the rate of guanine nucleotide exchange [88]. The interactions between proline-rich sequences in the COOH-terminal region of Sos and the SH3 domain of Grb2 or E3b1 appear to serve as a device to segregate Sos into two distinct functional pools: one that catalyzes guanine nucleotide exchange on Ras and another that stimulates the guanine nucleotide exchange on Rac [89] (Figure 1).

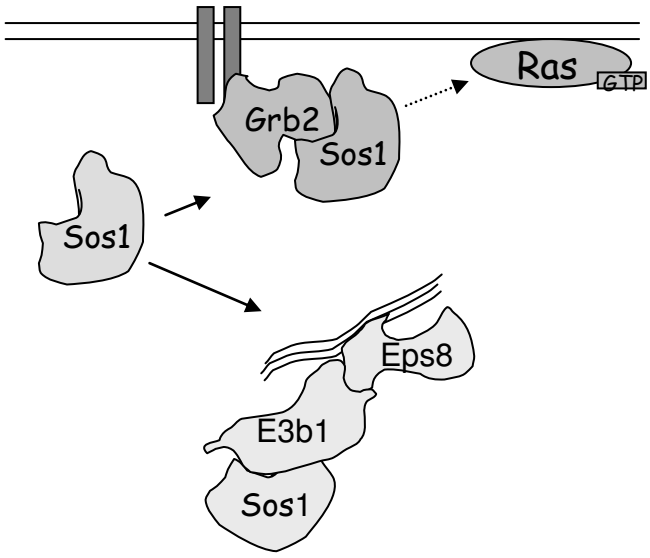

Fig.1 Upon Growth Factor stimulation, Sos is able to act together with Grb2 as a nucleotide exchange factor for Ras or is recruited to actin filaments together with E3b1 and Eps8 proteins and inducing then Rac activation.

2.2.2.1 Ras

The Ras protein was discovered in the 1980s and it was soon evident that it plays a critical role in controlling cell growth, Therefore Ras was one of the most intensively studied proteins of the past decade [90, 91]. Ras is a GTP-binding protein protein of around 170aa and like most of the transforming genes, Ras members were found by transforming NIH 3T3 cells with genomic DNA from different tumors [92]. The first Ras family member discovered is encoded by the v-H-ras gene and is the homologue of the transforming gene of a bladder carcinoma cell line (T24), the oncogene of the Harvey sarcoma [93, 94]. Afterwards it was discovered that the transforming gene Lx-1 of a human lung carcinoma cell line, is a human homologue of v-K-Ras, the oncogene of the Kirsten sarcoma virus [95]. One year later Shimizu *et al.* recognised the third and last member of the ras family, N-Ras-1 gene, the transforming gene of the SK-N-SH neuroblastoma cell line [96]. These three members of the Ras superfamily are structurally similar [91], but in reality the real number of this family members is not really clear since some authors argue about the presence of totally five

members (H-ras-1 and 2, K-ras-1 and 2, N-ras-1) [97], while other authors describe four true Ras protein (H-ras, K-rasA, K-rasB, N-ras) [98]. Ras is attached to the cell membrane by prenylation, and is a key component in many cell signaling processes that at the end lead to cell growth and proliferation. Ras proteins are present in resting cells in an inactive form, a guanosine diphosphate (GDP)-bound state. Guanine nucleotide exchange factors (GEFs) induce Ras to release GDP and bind GTP, which causes its activation and the subsequent stimulation of downstream targets. Having performed these functions, GTPase activating proteins (RasGAPs) turn Ras off, inducing the hydrolysis of GTP to GDP [99, 100]. The binding state of Ras is basically regulated by two protein families, GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) like Sos that induce GDP-GTP change. The activation of a tyrosine kinase receptor recruits Grb2 and Sos. In this conformation Sos is then able to bind to Ras through its CDC25 domain [101]. GEFs and GAPs play the role of accelerating a normal GDP-GTP exchange process. The mechanism by which they work has been a matter of debate [102-105].

Activation of Ras requires translocation of the protein to the plasma membrane, where the signal start [106, 107]. Ras anchorage to the plasma membrane is possible due to post translational modifications, like prenylation, isoprenylation or lipidation, that result in the addition of hydrophobic groups. It is usually assumed that prenyl groups facilitate attachment to cell membranes, similar to lipid anchors like the glycosyl-phosphatidylinositol anchor (GPI). Some reports suggested the possibility that Ras induces signals not only from the plasma membrane but also from the Golgi or from the endoplasmic reticulum [108].

All these evidences where found in cells where H-Ras was artificially overexpressed, in order to mimick the situation present in tumor cells [108, 109]. Oncogenic Ras was frequently found in different tumors but the gene mutated (H-RAS, K-RAS or N-RAS) varies widely depending on the tumor type [110]. K-RAS is however the most frequently mutated gene in tumors, for this reason expecially in breast cancer, downstream protein along Ras cascade, like p21 $^{WAF1/CIP1}$, have been related to factors of established prognostic significance, and to clinical outcome after primary treatment in terms of disease-free interval and survival times [111, 112]. In fact, the contribution of aberrant Ras function to human malignancies is likely to be higher than that indicated by Ras mutation status, as the over expression of many tyrosine kinase growth factor receptors also leads to increased Ras-dependent signaling [110]. Like the normal Ras protein, the Ras oncoprotein is activated by binding GTP, but is then less

likely to be inactivated. Indeed the oncoprotein, due to point mutations was found to have lost virtually

all GTPase activity [106]. In such a condition, it could be pushed into its active signal-emitting configuration by upstream stimulatory signals and guanine nucleotide exchange factors and once in this activated state, the Ras oncoprotein is unable to turn itself off [113].

Most of Ras mutants were identify in that state and the gene mutations more often found are in the 12^{th}, 13^{th} and 61^{st} amino acid of the protein, located around the cavity where the GTPase catalytic activity operates [114-116]. When the signal cannot be switched off, cells are continuously stimulated for growth and proliferation and specific downstream proteins are induced to transduce the signal.

In general, Ras effector proteins are characterized by presence of a Ras-binding domain also called RBD. At least three distinct RBDs have been recognized:

- the RBD of RAF involved in the cascade of the MAP kinase [117].
- the RBD from PI3K the first kinase of the PI3K/AKT pathway [118].
- the RAS association (RA) domains of RalGDS and ARF proteins [119].

2.2.2.2 MAP kinase, RAF/MEK/ERK pathway

This pathway is only one of the several downstream signaling cascades activated by the GTP-bound Ras protein. Soon after its discovery, over two decades ago, its important role in cell signaling was immediately evident [90]. In fact, antisense and dominant-negative Raf expression blocked tumor cell transformation induced by the RAS oncogene in cell culture [120, 121]. Raf was discovered by two groups independently as a retroviral oncogene, v-Raf or v-Mil, possessing a serine/threonine kinase activity [122-125]. Three mammalian Raf isoforms exist and are called A-Raf, B-Raf (BRAF), the most potent activator of the MAPK pathway and C-Raf the first to be identified and ubiquitously expressed [126]. The three mammalian Raf isoforms share domains of structural homology, designated CR1 (conserved region-1), CR2 and CR3 even if they present several functional variations. CR1 is located at the N-terminal region of the protein and contains two important domains both responsible for Raf membrane recruitment. The first is called RBD (Ras binding domain) and is responsible for the binding of Raf to Ras-GTP. The second common domain is defined as a zinc finger structure or cysteine-rich domain (CRD) that interacts with the lipid moiety on Ras. This binding gives a conformational activation signal to the RAF kinase domain [127-130]. The CR2 domain is the regulatory binding site and finally the CR3 domain is the C-terminal kinase domain that also contains a second binding site for regulatory proteins. As mentioned above the central role of Raf in tumorigenesis was immediately clear and now several different forms of Raf oncogenes are known. Among the 3 Raf members, BRAF is the most potent activator of the MAPK pathway in many cells [131]. Therefore, activating mutation in the *BRAF* gene are of particular importance in human cancers, especially in those that show a high prevalence of this mutation like melanma.

Some of Raf family members present point mutations or a more severe deletion in the N-terminal regulatory region, resulting in a constitutively active kinase. Thereby this activation results in a constitutive activation of its downstream targets MEK (MAPKK) and ERK (MAPK) [132-134]. Approximately 30% of all human cancers display evidence of constitutive activation of the Raf/MEK/ERK pathway.

The dual specificity kinases MEK1/2 phosphorylate ERK1/2 on a tyrosine and a threonine residue leading to their activation. About 160 substrates have been described for ERK1/2, many of them localized in the nucleus and involved in the transcription of genes [135].

The role of these downstream kinases are crucial for tumor growth and the Ras signal cascade. In fact transgenic mouse expressing Ras oncogene or overexpressing Erk proteins present a similar phenotype [136].

One important parameter that participates in the regulation and function of MAPK signaling is the subcellular localization of the protein cascade members [137]. It was shown that both ERKs and MEKs are localized in the cytosol of resting cells and that they translocate into the nucleus upon cellular stimulation [135]. However, while ERKs remain in the nucleus of the stimulated cells for up to 180 min, MEKs are rapidly exported out of the nucleus due to their nuclear export signals (NES). In the same region of NES, the MAPK-docking domain, responsible to bind and sequester ERK in the cytoplasm is also present. More precisely this domain recognizes the CD domain of the inactive form of ERK [138, 139].

All of these docking sites are located outside the catalytic domain and determine the specificity of interacting molecules. In quiescent cells, MEK1/2 retains ERK1/2 in the cytoplasm through direct interaction [138]. Upon stimulation, ERK1/2 becomes phosphorylated at threonine and tyrosine residues and the latter results in a conformational change and in the dissociation of ERK1/2 from MEK1/2. ERK1/2 then translocates to the nucleus by three mechanisms: passive diffusion of a monomer, active transport of a dimer, and direct interaction with the nuclear pore complex [140, 141]. In the nucleus, ERK phosphorylates and activates several nuclear targets such as transcription factors. This nuclear location seems to be an essential prerequisite for proper cellular responses. After activation of its target, ERK must relocalize to the cytoplasm thereby MEK1/2 transiently enters the nucleus by passive diffusion and export ERK1/2 from the nucleus [142, 143]. The nuclear localization of MEK1/2 is also regulated by a rapid stimulus-dependent transport mechanism [143, 144]. As already mentioned above, several studies *in vitro* and *in vivo* have proved the essential role of MAPK pathway in normal cell proliferation and migration, thereby in tumor formation and development. For this reason, several approaches were employed to induce or silence the signals of each member of this pathway [145].

In general, whenever Ras activates a MAPKKK protein it is possible to identify it by monitoring the activation state of each of the downstream protein, as MAPKK and MAPK, since the activation of one member lead to the activation of the only identified direct downstream target (Figure 2).

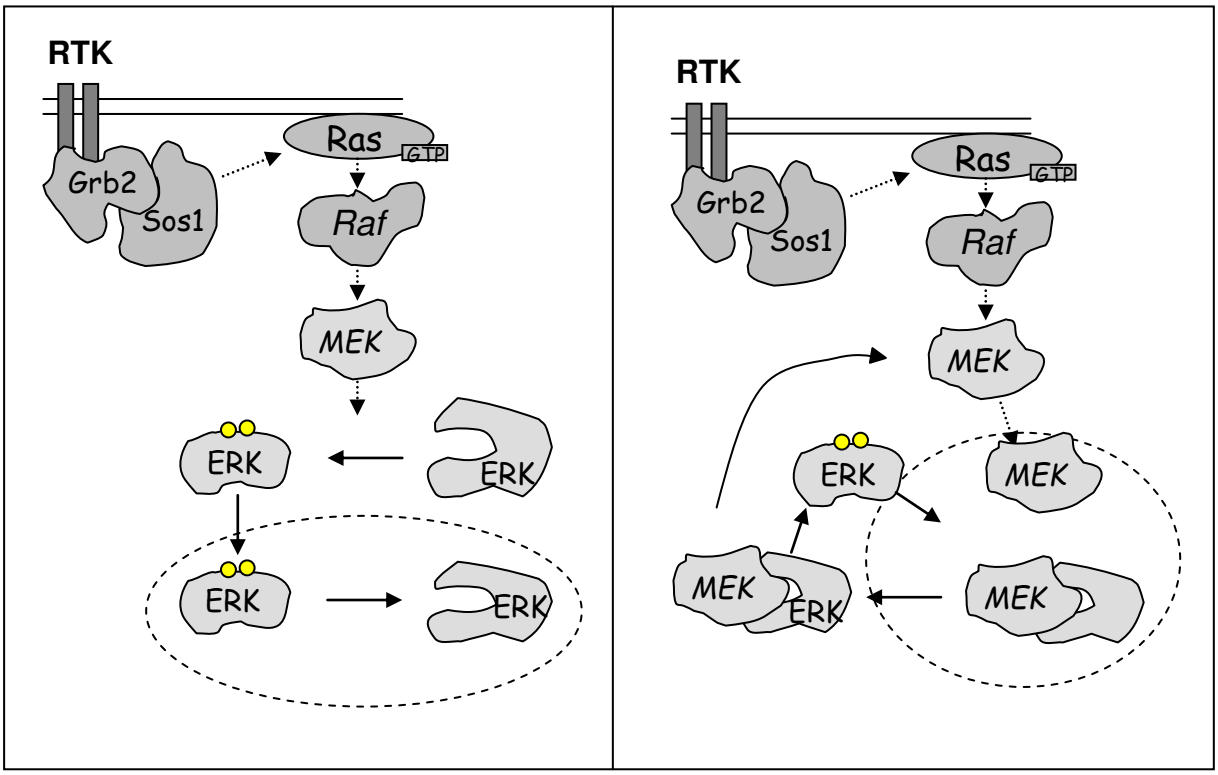

Fig. 2 Activation of the ERK signaling pathway. In the nucleus, ERK induce the transcription of several nuclear targets. When ERK activity is turned off, MEK enters the nucleus, binds to inactive ERK and brings it back to the cytoplasm.

2.2.2.3 PI3K/AKT kinase pathway

Early studies on the plasma membrane identified the presence of membrane-associated phospholipids with a purpose fully unrelated to the maintenance of membrane structure [146]. Further studies, demonstrated that some of these phospholipids contain an inositol ring at their hydrophilic moiety (phosphatidylinositol, PtdIns) that can be modified by the addition of a phosphate group, producing a phosphoinositol ring [147, 148]. PtdIns consists of a D-myo-inositol-1-phosphate linked via its phosphate group to a diacylglycerol tail that lies within the inner leaflet of the lipid bilayer of cells playing a role in anchoring the PtdIns to the cell membrane [147, 148]. The inositol ring presents five free hydroxyl groups and three were found be phosphorylation sites. Further evidences demonstrated that this phosphorylation was a crucial point for the beginning of a cascade of signals involved in important cellular functions like apoptosis, cellular proliferation [149], vesicular trafficking, cytoskeletal

organization and change in cell morphology [150]. The inositol moiety of phosphoinositides can be phosphorylated by several distinct kinases, each of which shows specificity in phosphorylatin a particular hydroxyl group of the inositol ring [151]. While several PI kinases have been discovered, the most important of these in cancer research is PI3K that is responsible for attaching a phosphate group to the 3'-hydroxyl group of the inositol moiety. The substrate of PI3K can be PtdIns or a phosphatidylinositol already phosphorylated in some of the other hydroxyl groups. Until now, there is evidence of only three different substrates: PtdIns, PtdIns4P and PtdIns(4,5)P_2.

The various 3-phosphorylated phosphoinositides that are produced by PI3k (PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3) function in a mechanism by which a selected group of signalling proteins, containing PX (phox homology) domain, pleckstrin homology domains (PH domains) and other phosphoinositide-binding domains, are recruited to the membrane and activated in order to transmit the signal downstream along the cascade [151]. This enzyme can be activated directly by cell surface receptors. In fact when a growth factor binds to the extracellular domain of an RTK, the receptor dimerizes and activates its cytoplasmic kinase activity. The autophosphorylated cytoplasmic domain of the RTK is a binding site for Src homology-2 domain-containing proteins. This is the case of PI3K that through its SH2 domains binds to the receptor and is thereby activated [152]. PI3K was demonstrated to be activated also by other cytoplasmatic proteins like Ras [153, 154]. Other reports also recognized other proteins that present either the SH2 domain or the SH3 domain and are able to transduce directly the signal from the RTK to the PI3K. A particular family of tyrosine kinase called Src were demonstrated to interact with PI3K even if this is not fully understood yet [155]. On the base of sequence homology and substrate preference *in vitro*, PI3K isoforms are divided into three different classes: class I, class II and class III.

-<u>class I</u>: These PI3K enzyme members are composed of a catalytic subunit and an adaptor or regulatory subunit. Their preferred substrate *in vivo* is only PtdIns(4,5)P_2 but *in vitro* they are also able to phosphorylate PtdIns and PtdIns(4)P. This class of enzymes presents catalytic subunits that bind to several regulatory subunits [156]. Catalytic subunits can be subdivided in subclass I_A (including three members: p110α, p110β and p110δ), which are regulated by binding to RTKs and subclass I_B (which contains only one member p110γ) that transduces signals originating from G-protein-coupled

receptors [157, 158]. The class I_A catalytic subunits bind to the regulatory subunits (that includes p85α, p85β, p55γ and p101) forming a heterodimeric complex [159].

Subclass-I_A is coupled with the p85α and β subunits, while p110 γ is coupled with p101 or p84 regulatory subunit [159, 160]. The catalytic PI3K subunits (p110α, p110β, p110δ and p110γ) present the Ras binding domain, similar to the RBD found in Raf (already described previously) that is responsible for binding to Ras and activating the lipid kinase activity. The other important domains present in these subunits are the catalytic domain responsible for binding ATP and PtdIns, which is able to specify substrate preferences and a C2 domain, a protein domain of 80-160 amino acid that posseses phospholipid-binding property. It was first recognized in PKC molecules and confers the phospholipid binding property to the protein. The C2 domain is thought to be involved in calcium-dependent phospholipid binding and in membrane targeting processes such as subcellular localisation [161]. Protein presenting this domain are involved in signal transduction or membrane traffic [162, 163]. The regulatory p85 subunits, on the other hand, present an SH3 domain and a structure of two SH2 domains separated by an inter-SH2 region, which serve as a docking site for the catalytic p110 subunit [159, 164].

Another important difference that is worth to be mentioned about the catalytic subunit is that p110α and p110β are widely present in all the human cells.

-class- II: Consist in three mammalian members, C2α and C2β, and C2 γ of about 170kD. As the name already underlines, the characteristic of this class is the presence of two C2 domains, rather than only one as in the class I PI3K members [165].

The second carboxyl-terminal C2 domain seems to possess different properties in comparison with the other C2 domain also present in the other PI3K classes.

Enzyme members of the class II also present a kinase domain responsible for PtdIns phosphorylation. Differently from class one, these proteins are composed by one subunit and they do not seem to bind any regulatory subunit. The members of this class have different preferences for the substrate compared to class I enzymes, PI3KC2α and C2β seems to prefer PtdIns but also act on PtdIns(4)P.

-class-III: Class III are similar to II in that they bias the production of PI(3)P from PtdIns, but are more similar to Class I in structure, as they exist as a heterodimer of a catalytic

(Vps34 homologue) and a regulatory (p150) subunit. Recent studies suggest that this class of PI3K is involved in diverse intracellular trafficking events including autophagy [166] and phagosome formation [167], internal vesicle formation within multivesicular endosomes [168] and transport at the nuclear membrane [169].

PI 3-phosphorylation is a balance of the action of PI3K and a phosphatase called PTEN (Phosphatase and Tensin homolog) that is able to dephosphorylate PtdInd(3)P thereby stopping the cascade of signal. PTEN is a protein that acts as a tumor suppressor gene and is found in almost all tissues in the body. The PTEN protein acts as a phosphatase to dephosphorylate phosphatidylinositol (3,4,5)-trisphosphate (PtdIns $(3,4,5)P_3$). The product of this enzymatic reaction is PtdIns$(4,5)P_2$. This dephosphorylation is important since it results in inhibition of the AKT signaling pathway [170]. The structure of PTEN (solved by X-ray crystallography) reveals that it consists of a phosphatase domain, and a C2domain. The phosphatase domain contains the active site which carries out the enzymatic function of the protein, whilst the C2 domain allows PTEN to bind to the phospholipid membrane so that it is able to de-phosphorylate PtdIns $(3,4,5)P_3$ [171].

Once activated by Ras or RTKs, PI3K phosphorylates the inositol ring of PtdIns. There are some proteins able to recognize and bind 3´-phosphoinositides, through two domains that are the FYVE zinc domain (the name derives from the four proteins where it has been found in: Fab1, YOTB/ZK632.12, Vac1, and EEA1) (80-90 aa) and the PH domain (100-120aa). The PH domain is a domain able to bind to phosphoinositides and their derivatives and was found in more then 100 different molecules [172-174]. The most important PH containing protein is AKT also called PKB. Once PI3K phosphorylates the inositol ring of PtdIns, AKT binds it through its PH domain and in this way it can also be phosphorylated and therefore activated. AKT/PKB is a serine/threonine-specific protein kinase and once activated proceeds to phosphorylate a series of protein substrates that have multiple effects on the cell as described below [175, 176]. AKT is a family composed of three members, AKT1, AKT2 and AKT3 that present very similar sequences and sites of activation. The binding of AKT to PtdIns(3,4)and PtdIns$(3,4,5)P_3$ is possible through the PH domain [177].

In the AKT amino acid sequence two threonines and two serines were identified as possible sites of phosphorylation. Of these Thr308 and Ser473 were shown to be important for activation and are located, respectively along and after the kinase domain [178]. On the contrary the Thr450 and the Ser124 phosphorylation, located, respectively before (N-

terminal) and after (C-terminal) the kinase domain are not essential for the activation of the enzyme. Recent observations suggest that Thr450 controls the correct folding of the protein, then AKT is translocated to the plasma membrane and finally in the third step the enzyme is activated by Thr308 and Ser473 phosphorylation [178]. More precisely when PIP_3 levels are elevated, AKT is recruited to the plasma membrane and phosphorylated in the "activation loop" (Thr308) by PDK1. In addition, AKT contains a highly conserved C-terminal hydrophobic motif (HM) that must also be phosphorylated for AKT activation [179]. Recent studies have indicated that TOR (target of rapamycin) complex 2 (TORC2) is responsible for HM phosphorylation [180, 181], implying a critical role for TORC2 in regulating AKT signaling and tissue growth. The kinase activity of TORC2 is provided by the protein kinase TOR, which is also found in a second complex, TORC1.

Once activated by these phosphorylations, AKT/PKB proceeds to phosphorylate and activate a series of protein substrates that have multiple effects on the cell.

The major biological effects that Akt/PKB has in cells are:

- **participating in cell survival by reducing the activation of the cellular apoptotic suicide program**.

  The most important family of proteins involved in the control of apoptosis is called Bcl-2. The Bcl-2 family members are divided into 2 classes depending on their role in apoptosis, pro-apoptotic (Bax, BAD, Bak) or anti-apoptotic (including Bcl-2, Bcl-X_L, and Bcl-w, among an assortment of others). They form heterodimers sequestering each other and, depending on which proteins are predominant in the cytoplasm, the apoptotic stimulus can start [176]. In this model if the pro-apoptotic proteins are the majority, they form homodimers, creating pores in the mitochondrial membrane through which cytocrome c is released [182]. This event, considered an early key event in apoptosis, acts as an essential cofactor in the cleavage and activation of the pro-caspase-9. Once activated, caspase-9 initiates the caspase cascade and activates the executioner caspases [183]. Akt/PKB has been shown to inhibit cytochrome c release from mitochondria and thereby prevents initiation of the apoptotic cascade that lead to activation of caspases [184]. Akt is also able to phosphorylate the proapoptotic protein BAD, thereby inactivating it and inducing the release of the anti-apoptotic proteins allowing them to inhibit apoptosis [185]. Recently, the ability of AKT to phosphorylate and inhibit the activation of the pro-caspase-9 was also demonstrated [183]. Another important potential mechanism that Akt utilizes to regulate

this cascade is transcriptional control of the proteins involved in it, via its recently described effects on forkhead (FKHR) and possibly on NF-*k*B transcription factors [186].

- stimulating cell proliferation.

The PI3K/AKT pathway was demonstrated to be involved in inducing cell cycle progression [187]. Cytoplasmic relocalization of nuclear proteins is a common phenomenon and has become as an established mode for their functional inactivation. It has been shown that AKT is able to phosphorylate $p27^{kip1}$ and $p21^{WAF1/CIP1}$ proteins [188] and Akt-induced phosphorylation of $p27^{kip1}$ and $p21^{WAF1/CIP1}$ causes retention of them in the cytoplasm, thereby inactivating their ability to inhibit cell cycle [189-191].

- Regulation of protein translation and cell growth

AKT was shown to be able to activate a central protein called mTOR, also known as FRAP, RAFT1 or RAPT [192]. mTOR is a protein composed by several distinct domains like the N-terminal region containing 20 tandemly repeated HEAT (Huntingtin, elongation factor) recognized responsible for protein-protein interaction and also in plasma membrane localization of the protein [193, 194]. Then there is FAT domain that serves as a scaffolding domain, followed by the FKBP-rapamycin binding domain, the kinase catalytic domain and a C-terminal FATC domain [195].

The most important downstream targets of mTOR identified are S6K and eIF-4E BP1 that respond in tandem to mTOR activation. S6K protein is present in two isoforms in mammalian cells (S6K1 and S6K2) and encoded by two separate genes [196]. A large amount of evidence suggests that once mTOR activates S6K, by phosphorylating it, this protein takes part in the control of cell growth via increasing mRNA translation [197].

The generally accepted model is that S6K activation causes increased translation of 5' terminal oligopyrimidine tract (TOP) mRNAs, which encode components of the translational apparatus, including ribosomal proteins, elongation factors, and poly(A)-binding protein (PABP) [198, 199]. Important ribosomal proteins that have been shown downstream S6K, are the S6 protein and eIF-4E pathway [200], They play the role of facilitating the binding of the ribosome to the 5'-mRNA end and then increase protein translation [201].

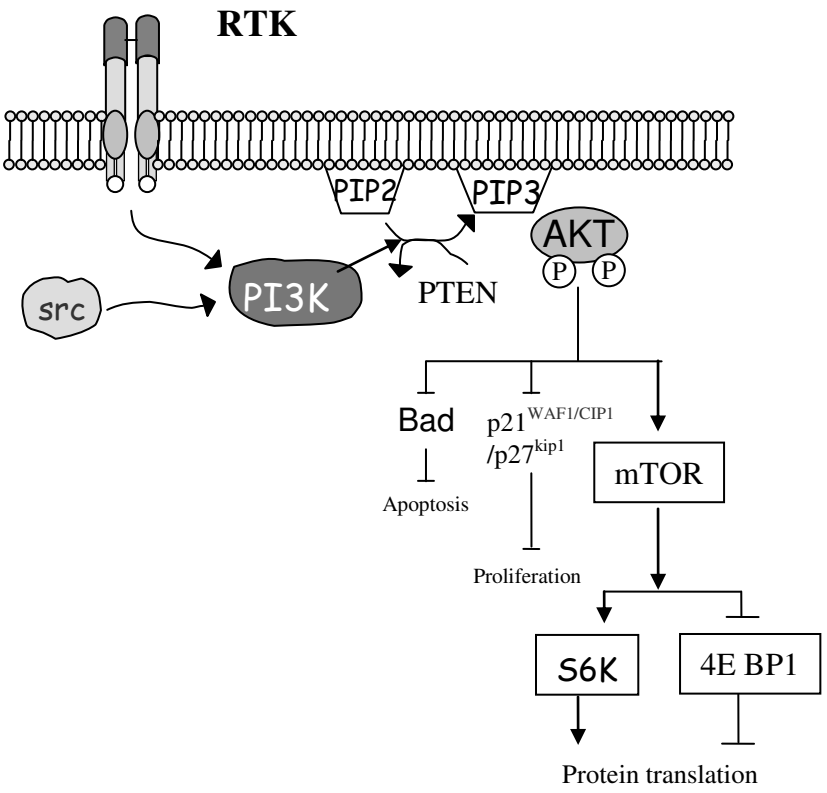

Figure 3. PI3K is activated directly by the RTK or by Src. After activation, PI3K is able to induce PIP_3 formation and then activation or silencing of several downstream proteins involved in cell proliferation, apoptosis or protein translation.

All the kinases presented until now as downstream of the PI3K/PTEN/AKT pathway, are just part of what seems to be involved. In fact new papers come out every day reporting about new roles for PI3K and AKT. Moreover, there is evidence that other than AKT/PKB other cytoplasmatic proteins use their PH domains to associate with PI(3)Ps, and that this association also induces their functional activation [202]. Among the PH-bearing cellular proteins are a group of guanine nucleotide exchange factors (GEFs) that act analogously to Sos, being responsible for activating various small GTPases that are relatives of the Ras proteins. These others GTPases belong to the Rho family of signaling proteins, which most well known members include Rho protein and its two relatives, Rac and Cdc42 [203, 204].

Like Ras, these Rho operate as binary switches, cycling between a GTP-bound active signaling state and GDP-bound inactive state [205]. The Rho-GEFs, once activate, act on Rho proteins in the same way that Sos act on Ras. In particular, the Rho-GEFs induce Rho proteins to exchange GDP to GTP [206]. Once activated, Rho proteins have functions that differ strongly from those of Ras: they participate in reconfiguring the structure of the cytoskeleton and the attachments that the cell makes with its physical surroundings. Recently some authors have investigated the role that Ras could have in Rho-GEFs activation [207]. From what is evident until now, Cdc42 and Rac work in tandem in inducing the remodeling of actin filament at the migration front [208].

Rho also functions in the contractile actin-myosin filament assembly. This activity in migrating cells is located in the back tail where through contraction can induce the removal of the backfront (stress fibers). Rho Is also involved in the formation of focal adhesion complexes involved in the anchorage of the cell to the surrounding [209].

Rac and Cdc42 on the other hand were shown to be located at the front of migrating cells and are involved in the formation of cytoskeletal elongations like filopodia for Cdc42, and lamellipodia, ruffles for Rac [210-212].

According to this, Rho and Rac/cdc42 play opposite roles in cell migration and are also able to mutually regulate their activity. Mammalian Rac exists in 3 different isoforms, but most of the studies were done on Rac1 that is expressed ubiquitously, rather than Rac2 present in hematopoietic cell, and Rac3 present in the brain cells [213]. Furder explanations regarding this pathway are present in the section 2.5.

2.3 PI3K signalling in breast cancer

The progression of primary mammary epithelial cells to a malignant phenotype involves multiple genetic events including activation of oncogenes and inactivation of specific tumor suppressor genes. In this regard, activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is thought to play a critical role in both the initiation and progression of human breast cancer. Activation of PI3K pathway can occur in response to a variety of stimuli that through the engagement of cell surface receptors then induce PI3K pathway activation. The active receptor triggers the recruitment of p85 regulatory subunit that then activates the catalytic PI3K subunit p110 [214].

Until few years ago, there were no evidences for activating mutations directly in PI3K genes. Therefore, this model could explain overactivation of this pathway and tumor formation. An increasing body of evidence, suggest that direct activation of the PI3K pathway can occur via activating mutations in the catalytic p110α subunit and this may play a critical role in the regulation of malignant breast cancer growth [215].

2.3.1 *PI3KCA*- Mutations in the *PI3KCA* gene, which encodes for the class I p110α catalytic subunit, were recently discovered in many different human cancers including lung, brain and breast tumors [216-218]. Many authors reported about amplifications, deletions and more recently, somatic missense mutations, all increasing the kinase activity of p110α [219]. All these mutations seem to be located preferentially in exons 6, 9 and 20 that code for the kinase, helical and p85-binding domains [219, 220]. The first study described *PIK3CA* gene mutations in 30% of colorectal, brain and gastric cancers but only in a small percentage of breast cancers [216]. More recent data that considered a larger variety and number of primary breast cancers and breast cancer cell lines, reported that 70% of the samples presented mutations in the *PI3KCA* sequence [217, 221]. But this point is still a matter of discussion since more papers in accordance with either the preliminary data or the more recent data come out daily [222]. Together with this, there is another matter of investigation which results are extremely controversial. In fact some authors suggest that there is no association between the presence of *PI3KCA* mutations with other prognostic/clinical features of breast cancers, including histological subtype, oestrogen/progesterone receptor expression, Her2/neu receptor status, axillary lymph node

positivity and grade or stage of the tumor [221, 223]. This is in contrast with some others evidence reported recently [224]. In general the only point that seems to put everybody in agreement is that considering all the PI3K members, their alterations are present in most of the breast cancers [225]. All these controversial results are likely due to a number of factors including geographical influence, sample source, methods for DNA extraction and the stage of the tumor sample. Anyway more recent data, produced with new techniques and with breast cancer samples chosen with a higher accuracy, proved high frequence of *PI3KCA* gene mutations in breast cancer and a correlation with tumor stages. However, despite these discrepancies the high frequency of *PI3KCA* mutations and the discovery of hotspot mutations have important clinical implications for diagnosis, prognosis and therapy, in fact therapies targeted specifically against mutant *PI3KCA* can be developed [226, 227]

2.3.2 *PI3KCB*- Not much is known regarding the role and the existence of mutations of this class I_B protein kinase in cancer. Some authors demonstrated that there is a copy number alteration in more than 20% of late-stage primary ovarian cancers [228] but other mutations in this gene have not been reported. Its silencing with siRNA was decreasing tumor growth in glioblastoma, proving its crucial role in tumor progression [229]. There are no studies regarding mutations in *PI3KCB* and roles in breast cancer.

2.3.3 *PI3KCD*- There are no evidences of mutation in this gene in breast cancer, some evidence and knowledge of over-expression are coming out now in neuroblastoma [230].
Regarding breast cancer, the only observation already published indicate that p110□ confers a higher migratory capacity to breast cancer cells [231].

2.3.4 *PTEN*-This phosphatase is responsible for turning off the signal produced by PI3K. This means that together with activating mutations of PI3Ks, inactivating mutations of PTEN have also been frequently reported in cancer cells. The first evidences for mutations in PTEN came from investigations regarding the hereditary cancer predisposition Cowden syndrome (CS). This syndrome causes a huge amount of disturb but the most important was their high risk for developing cancers of breast and thyroid [232]. The genetic locus for Cowden syndrome was mapped to the 10q23 region that is the same where also PTEN gene is located [233]. PTEN mutations were discovered in 80% of the CS patients and from this a more accurate analysis of the locus revealed germline mutations in a great amount of tumors

[234]. In this locus many somatic deletions frequently found in tumors were described. In breast cancer, prostate cancer and glioblastoma biallelic inhactivation of PTEN, loss-of-heterozygosity and point mutation in sometimes more then 50% of the tumor samples were reported [235, 236].

2.4 TGFβ Receptor

Transforming growth factor-β (TGFβ) was first identified as a component of sarcoma growth factors, secreted from Monoley sarcoma virus infected 3T3 cells that could mediate transformation (anchorage-independent cell growth) of non-neoplastic rat kidney NRK and murine AKR-2B fibroblasts [237]. The TGFβ receptor controls a diverse set of cellular processes, including cell growth, apoptosis, differentiation, migration, extracellular matrix (ECM) production, angiogenesis, immunity and epitheliomesenchymal transition (EMT) [238-240]. TGF family receptors are related to the tyrosine kinase reseptors, in that they have an extracellular ligand-binding domain, a transmembrane anchoring domain and a cytoplasmic serine/threonine kinase domain. It is important to mention that they have a substantial difference compared to RTKs receptors. In fact TGFβ kinase phosphorylates serine and threonine rather than tyrosine residues [241]. The TGFβ superfamily of growth factors consist of TGFβ members (1, 2, 3), Activins, Nodal and Bone Morphogenic Factor (GDF) and a few other minor members like BMP [242]. Regarding the receptor family there are only three members that are called type I, type II and type III. Among them only type I and II receptors are able to form heteromeric complexes and initiate intracellular signaling following ligand binding [241]. Until now 7 different mammalian type I and 5 different type II receptors are known and it seems that the heterodimers formed is different in depending of the specific grow factor activation [242, 243]. The type II receptor is constitutively phosphorylated on various serines, while type I is characterized by a region rich in glycine and serine (GS region), exactly before the kinase domain [241]. The TGFβ growth factor is recognized and bound by the TGFβ type II receptor that then binds to the type I receptor. This leads to crossphosphorylation of the GS domain in type I receptor by the type II kinase receptor, which results in activation of the type I receptor kinase domain and initiation of downstream signaling and phosphorylation of the Smad signaling mediators [244].

The most well characterized mechanism whereby TGF-β superfamily members initiate signal transduction is via the phosphorylation and activation of the Smad proteins. Untill now eight Smad proteins are known in mammals that can be grouped into three classes based on function :

- The Receptor activator Smad (R-Smad: **Smad 1, 2, 3, 5, 8**) [242]
- The Common mediator Smad (Co-Smad: **Smad 4**) [242]
- The Inhibitory Smad (I-Smad: **Smad 6, 7**) [242]

The structure of Smad family members is very similar along the three classes. They contains three distinct regions, the Mad-homology domains 1 and 2 (MH1 and MH2) and a linker domain. The MH1 domain is located at the N-terminal region of the protein, while MH2 is at the C-terminal [245]. The MH1 domain is required to bind the DNA when translocated into the nucleus after activation. Actually Smad also needs to recognize and bind to other cellular factors (like Jun, TFE_3, Sp_1) to form transcriptional complexes which also bind to the DNA, and the binding region for these other transcription factors is through the MH1 domain [246, 247]. Normally this domain is composed approximately of 130aa, but in the case of the Smad1 this sequence is extremely small and thereby is unable to bind the DNA. The MH2 domain does not bind to the DNA and instead is a multifunctional region that mediates association with a wide variety of proteins. Some of them serve to provide specificity and selectivity to Smad function. In this region there is a basic pocket composed by basic amino acids that constitute a docking site for phosphorylated glycine/serine rich domains (GS domain) of type I receptors [248]. At the extreme C-terminus of the MH2 domain in R-smad, there is a SSXS (Ser-Ser-X-Ser) motif which is phosphorylated by activated type I TGF□ receptors [249, 250]. On the other hand, Co-Smad and I-Smad do not present this motif and thereby they can not be phosphorylated. R-smad phosphorylation is facilitated by other proteins including SARA (Smad anchor for receptor activation) and HRS/HGS (hepatocyte growth factor-regulated tyrosine kinase substrate) [251, 252]. Both these adaptor proteins contain the phospholipid-binding FYVE domain and function as adaptors to aid in recruitment of R-Smad to the TGFβ receptor complex. In the end, once the TGFβ II receptor bind to the specific growth factor, it form a complex with TGF-β I, phosphorylating its C-terminal domain [248]. This signal induces recruitment and phosphorylation of R-Smad members (Smad2 is the most commonly activated after TGF-β stimulation). Phosphorylated Smad2

then forms a heteromeric complex with an other MAD-related protein, Co-smad4, and accumulates in the nucleus [253, 254]. Once in the nucleus, Smad2 also bind to the Forkhead activin signal transducer (FAST) through the MH2 domain of Smad2. The Smad4 MH2 domain then binds to DNA at a site adjacent to that of FAST and thereby stabilizes DNA binding by the Smad-FAST complex that then induces specific gene expression [255]. TGF□ signals also via the MAPK pathways and several other reports demonstrated that the Smad and MAPK pathways have functional interactions [256]. In all the cases, TGF□ always functions as an antiproliferative factor inhibiting cell growth by induction of apoptosis and cell cycle arrest (through $p21^{WAF1/CIP1}$ and p15 protein upregulation and myc downregulation) [257]. In several tumors it was observed that cancer cells increase TGF□ production and this correlates with the severity of the tumor grade. Tumor-derived TGF□ can affect cells in proximity of the tumor, thus producing a microenvironment that promotes tumor growth, invasion and metastasis [258]. In many tumor, principally in breast cancers, TGF□ can act on the tumor cells directly, and regulates their capacity to proliferate and migrate.

Especially regarding the complex process of cancer metastasis, the role of this growth factor has been documented. Recent studies demonstrate that Smad signaling is required for these TGF-□-induced bone metastasis of breast cancer cells [259]. RNA-interference-mediated depletion of Smad4 in MDA-MB-231 breast cancer cell line inhibited bone metastasis in a mouse xenograft model, and this could be restored by ectopic expression of Smad4 [259]. Azuma *et al*. demonstrated that adenoviral overexpression of Smad7 in cultured breast tumor cells and in an *in vivo* mouse model of breast cancer inhibited the invasive phenotype of tumor cells [260]. In general Smad signaling contributes to the oncogenic activities of TGF-□ in cancer cells that no longer respond to the antiproliferative signals of TGF-□, suggesting that Smad pathway is as a potential target to inhibit the invasion of malignant tumors [261].

The TGF□ receptor as already mentioned before, is similar to tyrosine kinase receptors and seems to be able to also induce the signal cascades presented in the previous paragraph. But regarding the ability of TGF-β to activate Ras, PI3K and ERK there are still few informations [262] (For its description see 2.2.2.3).

2.5 Cell migration and metastasis.

Despite improvement in detecting and treating primary tumors, long-term survival is often compromised by the development of metastatic lesions in unrelated tissues. In breast cancer the metastasis more often recognised are in brain and bones [263].

In the design of cancer treatments, the use of some adjuvant in order to eradicate residual disease and reduce the risk of secondary tumors is increasing. Understanding the process of metastatic cell dissemination and tumor cell dormancy is therefore critical to design new effective treatments [264]. In breast cancer especially, metastatic dissemination of cancer cells requires the acquisition of properties that are not typically found in epithelial cells [265, 266]. These properties include enhanced migratory behavior of the cells. As already described before there is a signal pathway that goes through RAS-PI3K-AKT and is crucial in inducing cell migration. The most important downstream family proteins responsible for it are the Rho family GTPases (For description, see 2.2.2.3). Together with Rho proteins another crucial role is played by the extracellular matrix (ECM). In tissue stroma, fibronectin and other ECM proteins provide adhesive signals as well as steric blockage of cell movement, to biochemically and mechanically restrict the movement of neoplastic cells from their primary tumor sites. Following loss of E-cadherin expression and other cell-cell contacts, many tumor cells exhibit altered expression patterns for the ECM receptors, along with increased secretion of matrix metalloproteases, to remodell tissue metrices and invade the vascular system [265, 267]. All these changes together are defined as the epithelial to mesenchymal transition (EMT) and enhable neoplastic cells to escape from ECM-restrictions on motility. A widely expressed family of cell surface receptors responsable for cell adhesion to the extracellular matrix (ECM) and for cell-cell interaction are integrins [268]. All the family members are structurally composed by an αβ heterodimer [268]. Until now there are 14 □ subunit and 8 □ subunits known and they could in theory associate giving more than 100 heterodimers. The hetorodimers founded until now describe are a more restricted [134]. Upon binding to specific ECM components, integrins are activated and clustered into regions of the plasma membrane termed focal contacts, or focal adhesions,nwhich anchor the actin cytoskeleton to the underlying ECM [269]. The clustering and dissociation of integrin cytoplasmic domains triggers the recruitment of a large number of membrane and cytosolic proteins to form submembrane plaques, termed focal contacts or focal adhesion, and is dependent on associated actin organization [270, 271].

Besides direct interactions with integrin cytoplasmic domains, an increasingly extensive list of cytoplasmic molecules have been found to be localized to focal adhesions, many of which interact indirectly with integrins and/or actin microfilaments [272]. Some of these are regulatory kinases including PI3K, PKC and tyrosine kinases such as FAK (focal adhesion kinase), Src and all together they orchestrate downstream proteins crucial for migration: Rho, Rac, cdc42. The model for cell migration is a highly integrated multi-step process that coordinates protrusion of the membrane and actin cytoskeleton at the migration front, assembly of focal contacts with which to stabilize attachment in the direction of coordinated motion, disassembly and release of focal contacts at the cell rear, and contraction with which to drive the cell on (Figure 4) [273, 274]. While represented as a step-wise process, each steps are occurring simultaneously, and provide mechanical feedback to the other aspects, thereby integrating and coordinating these four separate activities [275]. In polarized migrating cells, active membrane processes, including broad, sheet-like lamellipodia and thin, spike-like filopodia, are constantly extending and exploring around the cell front. Both of these structures are abundant in actin and actin-associated proteins, and their formation is tightly correlated with ongoing polymerization of actin filaments. Indeed, actin filaments are intrinsically polarized with fast-growing “barbed” or “plus” ends against the membrane, and slow-growing “pointed” or “minus” ends opposite the membrane, and this inherent polarity is used to drive membrane protrusion. In lamellipodia, filaments form a branching dendritic network, whereas in filopodia they are organized into long parallel bundles [208, 276]. In both cases, actual membrane protrusion is driven by actin polymerization that is regulated by the actin-related protein (Arp) 2/3 complex and actin severing proteins, such as actin depolymerizing factor (ADF, or cofilin) and gelsolin. In lamellipodial actin networks, Arp2/3 complexes bind to the sides or tip of pre-existing actin filaments and nucleate filament branching [276]. Activation of Arp2/3 complexes is starting from Rac and cdc42. They activate downstream protein WASP/WAVE family members that then activate Arp2/3 proteins and are themselves localized to the cell membrane [208, 277]. Protrusions in filopodia are thought to occur by a filament repetitive mechanism, in which actin filaments within a bundle elongate at their ends towards the filopod tip and actin monomers are released at the pointed ends near the base. The distinct organization of these protrusions appear particularly well-suited to separate functions: localized activation of the Arp2/3 complex in lamellipodia can direct a wide movement of the cell body in a particular direction during migration, whereas the filopodial extensions are well-adapted as exploratory sensors. For

maintenance of movement by membrane protrusions and also to sustain the lamellipodia, extensions must be stabilized by the attachment of integrins and formation of bigger focal contact structures. Those at the front of migrating cells are small focal complexes, which are Rac/cdc42-dependent (through WASP/WAVE and then Arp2/3), highly transient, and facilitate rapid migration by generation of strong propulsive traction forces [278, 279]. Proteins like FAK and tensin were also identified early as components rapidly recruited to sites of integrin clustering [280]. FAK-Src signaling has been strongly implicated in turnover of focal complexes at the base of lamellipodia. Taken together, these studies underscore a central role for tyrosine phosphorylation in regulating protein-protein interactions critical to focal complex assembly and disassembly. While most focal complexes turnover at the base of lamellipodia, some undergo Rho-induced enlargement to form robust focal adhesions [281]. Focal adhesions are larger, elongated, take substantially longer to develop, are associated with stress fiber formation, and correlate with spreading and reduced motility. Differences in the molecular compositions between focal complexes and focal adhesions, aside from the involvement of Rho and some Rho effectors, are not well understood. Some observations suggest that Rho induces actin polymerization through a family of proteins, including DRF (diaphanous-related formin), mDia1 (mammalian homologue of diaphanous), and possibly mDia2. While Rho activity promotes focal adhesion maturation, its inactivation achived by Cdc42/Rac, appears to loosen adhesions, eventually leading to cell rounding [282, 283]. Coupling adhesion, protustion and actin organization are the myosin II-mediated contraction of the actin cytoskeleton [284]. This model incorporates a regulated actomyosin contractility and functional adhesion foci, which both generates force sufficient for locomotion and sufficient attachment to the ECM for the use of this force.

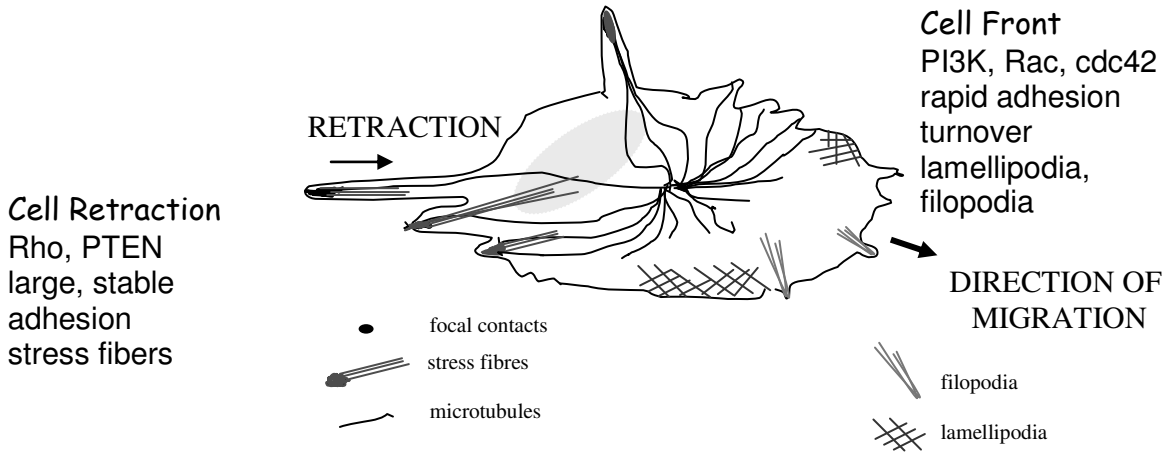

Fig.4 Cell migration and principal actors. During cell migration Rac and cdc42 proteins are located at the cell front and induce formation of filopodia and lamellipodia. Rho and PTEN are working in the opposite direction and are located in the cell retraction area, inducing formation and elongation of structure involved in cell adhesion, like focal adhesion, stress fibres and microtubules.

2.6 Src family tyrosine kinases.

Src is a family of proto-oncogenic tyrosine kinases originally discovered by J. Michael Bishop and Harold E. Varmus, for which they won the Nobel Prize [285].

Src family of kinases (SFKs) comprises at least 9 proteins Src, Fyn, c-Yes, Fgr, Lyn, Hck, Lck, Blk and Yrk, all approximately 60 kD in molecular weight and with high sequence homology [286, 287]. The best known and most investigated member of SFKs is Src, but there is considerable evidence to support the idea that all members perform redundant functions. Src is the transforming (sarcoma inducing) gene of Rous sarcoma virus. The protein product is a cytoplasmic protein with tyrosine-specific protein kinase activity that associates with the cytoplasmic face of the plasma membrane [288].

This kinase has several different domains: an N-terminal (or SH4) domain for membrane association, a unique domain, SH3 and SH2 domains for protein-protein interactions, a catalytic domain and a negative regulatory domain [288].

The inactive or closed conformation of this protein is achieved via phosphorylation of a conserved tyrosine residue (Y527 in avian c-Src), near the carboxyl terminal tail of the protein, which forms an intramolecular interaction with the SH2 domain [289]. Together with this there is also an interaction between the SH3 domain and the short sequence of amino acids linking the SH2 and the kinase domain [290]. This complex structure limits the accessibility to both the kinase domain and the SH3-SH2 domains limiting the activation of this protein. For their activation, Src kinases require to be phosphorylated on Tyr(416/419) within the activation loop [291].

Several reports identified Src as a kinase involved in signaling from many receptor tyrosine kinases, including EGFR, IGF-R, PDGF-R and others [292].

This evidence suggested that in general, Src family kinases, can promote signaling from growth factors receptors in a number of ways. In fact these proteins seems to be involved in several cell responses like DNA synthesis, cell proliferation, receptor turnover and actin cytoskeletal rearrangements during cell migration [293].

Since Src is involved in so many cell functions, it has bong been investigated and it has been found crucial in inducing proliferation, angiogenesis, metastasis and EMT [294]. Upregulation of Src family members was shown be important in many tumor types, like colorectal, breast and lung cancer [295]. Anyway several authors proposed that Src is able to associate with related adhesion focal tyrosine kinase (RAFTK , also known as Pyk2) cytoplasmatic protein and together phosphorylate RhoGAP and RasGAP proteins involved in actin and cytoskeletal rearrangements [296].

The outcome of Src binding to FAK (Focal Adhesion Kinase) is the phosphorylation of several tyrosine residues in FAK and FAK-associated proteins. FAK tyrosine phosphorylation sites that are targeted Src are several. Phosphorylation of tyrosine 925 has been mapped as a binding site for the SH2 domain of the Grb2 adaptor protein, which is believed to partially mediate activation of the Extracellular-regulated kinase (Erk) family of Mitogen Activated Protein (MAP) kinases [297]. The PI3K pathway has also been shown to be activated by Src. As already mentioned before, Src can be activated by several cell surface receptors, like EGFR. Recently authors demonstrated that EGF-stimulated epithelial cells migration requires the activation of a pathway dependent upon both PI3K and Src family kinases [298]. These and other data connecting Src activation to PI3K in migration, proliferation and other cell functions, are now starting to be published [299].

3 Results

3.1 Project Descriptions

3.1.1 The catalytic class IA PI3K isoforms play divergent roles in breast cancer cell migration

Breast cancer remains the most common malignancy among women in the Western world and patient mainly die from metastases rather than uncontrolled local disease. In this project we investigated the molecular mechanisms involved in the acquisition of a malignant invasive phenotype by breast epithelial cells in response to oncogenic H-Ras transduction and TGFβ I. We investigated the role of PI3K that from previous evidence appears to play a crucial role in this ability. We identified the PI3K p110α and p110β as proteins able to induce this response through the activation of pro-migratory signalling pathways (S6K, Cdc42/Rac).

Further investigations also identified PI3K p110□ as regulator of an anti-migratory pathway, involving AKT and Rho proteins. These results unveiled new roles for the members of PI3K family enzyme and suggest new possible targets and markers for metastasis.

In this manuscript I performed all the experiments aside from figures 8C and 8D (Olivier E. Pardo)

3.1.2 Phosphoinositide 3-Kinase C2β Regulates Cytoskeletal Organization and Cell Migration via Rac-dependent Mechanisms

Phosphoinositide 3-kinases (PI3Ks) trigger activation of signaling pathways in response to extracellular stimuli that regulate diverse cellular programs such as cell survival, proliferation and migration, phagocytosis, and glucose homeostasis.

Class I member were long investigated while instead the role of class II members is still unclear. In this report, we demonstrated that class II phosphoinositide 3-kinase C2β (PI3KC2β) associates with the Eps8/Abi1/Sos1 complex and is recruited to the EGF receptor as part of a multiprotein signaling complex also involving Shc and Grb2. Increased expression of PI3KC2□□stimulated Rac activity in A-431 epidermoid carcinoma cells, resulting in enhanced membrane ruffling and migration speed of the cells. Conversely, expression of dominant negative PI3KC2□□reduced Rac activity, membrane ruffling, and cell migration.

Moreover, PI3KC2□□overexpressing cells were protected from anoikis and displayed enhanced proliferation, independently of Rac function.
Taken together, these findings suggest that PI3KC2□ regulates the migration and survival of human tumor cells by distinct molecular mechanisms.

In this paper I performed the experiments in fig 9A and 9B.

3.1.3 Targeting PI3KC2β impairs proliferation and survival in acute leukemia, brain tumors and neuroendocrine tumors

While much attention has been given to the class I PI3K isoforms, little is known about the functions of class II PI3Ks in human cancer. This study was aimed at investigating the role of the PI3KC2β isoform in a panel of primary tumors and cell lines. Over-expression of PI3KC2β was detected in subsets of tumors and cell lines from acute myeloid leukemia (AML), glioblastoma, medulloblastoma, neuroblastoma, and small cell lung cancer (SCLC). Specific pharmacological inhibitors of PI3KC2β or small interfering RNA (siRNA) impaired proliferation of a panel of cell lines and primary cultures from AML, brain tumors and neuroendocrine tumors. Inhibition of PI3KC2β also induced apoptosis in AML and GB cell lines and sensitised the cells to chemotherapeutic agents. Furthermore, PI3KC2□□inhibition impaired the phosphorylation of downstream signalling mediators in AML. Together, these data show that PI3KC2β contributes to proliferation and survival in AML, brain tumors and neuroendocrine tumors and may represent a novel target in these malignancies.

My contribution to this manuscript is figure 7

3.1.4 Investigation of the role of Src Tyrosine Kinase in the regulation of PI3KC2β

PI3KC2β is a class II PI3-kinase family member able to phosphorylate the D3 hydroxyl group of a downstream target phosphoinositide. Several important pathways involve PI3KC2□ that once phosphorylated and thereby activated, transduce the signal, inducing different cell processes like proliferation, migration and actin rearrangement. From the sequence of this protein, 4 tyrosines were identified as possible site of phosphorylation (Y68, Y127, Y228, Y1541). Our previous studies proved a crucial role for Src proteins in activating PI3K class I members suggesting a possible second role for Src in regulating also Class II members. On the base of this observation we used HEK293 cells and proved that Src was able to interact with PI3KC2β increasing substantially its lipid kinase activity. With migration and proliferation tests, we then identified a crucial involvement of Src and PI3KC2β in inducing the response in HEK293 cells. We went on investigating which of the possible phosphorylation sites are important for PI3KC2β activation. We finally concluded that Src activates PI3KC2β, but when tyrosines 68 and 127 in the NH_2 terminal domain of PI3KC2β are mutated, the effect of Src on PI3KC2β activation was abolished in migration and proliferation assays. F-actin staining also confirmed that the mutants carrying phenylalanine instead of these two tyrosines were not able to induce the cytoskeletal organization that was evident in cells transfected with Src and PI3KC2β wild type.

3.2 Manuscripts

3.2.1 The catalytic class I_A PI3K isoforms play divergent roles in breast cancer cells (submitted to Oncogene)

The Catalytic Class I_A PI3K Isoforms Play Divergent Roles in Breast Cancer Cell Migration

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Running Head: PI3K isoforms in breast cancer migration

Abbreviations: CRIB, Cdc42 and Rac-binding region; EMT, epithelial to mesenchymal transition; Erk, extracellular signal-regulated kinase; GEF, guanine nucleotide exchange factor; MEK, mitogen-activated Erk kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome ten; S6K, ribosomal protein S6 kinase

ABSTRACT

Transforming growth factor-β (TGFβ) plays an important role in breast cancer metastasis. Here phosphoinositide 3-kinase (PI3K) signalling was found to play an essential role in the enhanced migration capability of fibroblastoid cells (FibRas) derived from normal mammary epithelial cells (EpH4) by transduction of oncogenic Ras (EpRas) and TGFβ1. While expression of the PI3K isoform p110δ was down-regulated in FibRas cells, there was an increase in the expression of p110α and p110β in the fibroblastoid cells. The PI3K isoform p110β was found to specifically contribute to cell migration in FibRas cells, while p110α contributed to the response in EpH4, EpRas and FibRas cells. Akt, a downstream targets of PI3K signalling, had an inhibitory role in the migration of transformed breast cancer cells, while Rac, Cdc42 and the ribosomal protein S6 kinase (S6K) were necessary for the response. Together our data reveal a novel specific function of the PI3K isoform p110β in the migration of cells transformed by oncogenic H-Ras and TGF-β1.

INTRODUCTION

The migration of cancer cells is one of the key factors responsible for cancer metastasis. The elucidation of mechanisms responsible for the highly invasive potential of cancer cells can help to identify specific targets for the treatment of cancer patients. Highly invasive cancers are usually characterized by aberrant activity of specific intra- or extracellular molecules such as protein kinases, phosphatases, transcriptional factors and proteolytic enzymes.

Phosphoinositide 3-kinases (PI3Ks) are a family of signaling enzymes which regulate a variety of important cellular functions, including proliferation, growth, cell cycle progression, apoptosis, migration, metabolism and vesicular trafficking (Engelman *et al.*, 2006; Garcia *et al.*, 2006). Since human cancer cells often display abnormal regulation of these cellular processes, the realization that PI3K signaling is disrupted at multiple levels has prompted researchers to develop targeted therapies against individual enzymes involved in this signaling cascade (Shaw and Cantley, 2006; Workman *et al.*, 2006; Zhao and Roberts, 2006; Vogt *et al.*, 2007). A family of PI3K isoforms exists and these enzymes are subdivided in three classes (I-III), based on sequence homology and *in vitro* substrate specificity (Vanhaesebroeck *et al.*, 1997; Vanhaesebroeck and Waterfield, 1999; Foster *et al.*, 2003).

The first identified downstream target of phosphoinositide 3-kinase (PI3K) in platelet-derived growth factor (PDGF)-stimulated membrane ruffling was Rac (Hawkins *et al.*, 1995; Hooshmand-Rad *et al.*, 1997). Class I_A PI3Ks are implicated in many cellular responses controlled by receptor tyrosine kinases (RTKs), including actin cytoskeletal remodeling. Within this pathway, Rac is a key downstream target/effector of PI3K. One possible candidate for this function is the Rac-activating complex Eps8-Abi1-Sos-1, which possesses Rac-specific guanine nucleotide exchange factor (GEF) activity (Innocenti *et al.*, 2003). It was shown that Abi1 (also known as E3b1) recruits PI3K, via p85, into a multimolecular signaling complex that includes Eps8 and Sos-1 (Innocenti *et al.*, 2003). The recruitment of p85 to the

Eps8-Abi1-Sos-1 complex and PIP_3, co-operate to unmask its Rac-GEF activity *in vitro* (Innocenti *et al.*, 2003). Moreover, they are indispensable for the activation of Rac and Rac-dependent actin remodeling *in vivo* (Innocenti *et al.*, 2003). Upon growth factor stimulation, endogenous p85 and Abi1 consistently colocalized into membrane ruffles, and cells lacking p85 failed to support Abi1-dependent Rac activation (Innocenti *et al.*, 2003).

Direct PI3K activation was sufficient to disrupt epithelial polarization and induce cell migration and invasion (Keely *et al.*, 1997). PI3K inhibition also disrupted actin structures, suggesting that activation of PI3K alters actin organization, leading to increased motility and invasiveness (Keely *et al.*, 1997). Integrin-mediated activation of PI3K was shown to promote carcinoma invasion by targeting Rac (Shaw *et al.*, 1997). Vav, a guanosine diphosphate (GDP)-guanosine triphosphate (GTP) exchange factor (GEF) for Rac that stimulates the exchange of bound GDP to GTP bound, was directly controlled by substrates and products of PI3K (Han *et al.*, 1998). PI3K also acts upstream of Tiam1, an activator of Rac (Sander *et al.*, 1998). Akt/PKB potently promoted invasion of highly metastatic cells, by increasing cell motility and matrix metalloproteinase-9 (MMP-9) production, in a manner highly dependent on its kinase activity and membrane-translocating ability (Kim *et al.*, 2001). The increase in MMP-9 production was mediated by activation of NF-κB transcriptional activity by Akt/PKB (Kim *et al.*, 2001). However, Akt/PKB did not affect the cell-cell or cell-matrix adhesion properties of the cells. These findings thus established Akt/PKB as a major factor in the invasive abilities of cancer cells (Kim *et al.*, 2001). Another study showed that PI3K is constitutively active and controls cell motility of highly invasive breast cancer cells by the activation of transcription factor, NF-κB (Sliva *et al.*, 2002). The urokinase-type plasminogen activator (uPA) promoter contains an NF-κB binding site, and uPA expression in MDA-MB-231 cells was induced by the constitutively active NF-κB (Sliva *et al.*, 2002). Cell migration was inhibited by overexpression of a dominant negative p85α, as well as by pretreatment of cells with wortmannin and LY294002 (Sliva *et al.*, 2002). Highly invasive MDA-MB-231

cells constitutively secreted uPA in amounts significantly higher than poorly invasive MCF-7 cells (Sliva *et al.*, 2002). Furthermore, inhibition of NF-κB markedly attenuated endogenous migration, and inhibition of PI3K and NF-κB reduced secretion of uPA (Sliva *et al.*, 2002). These data suggest a link between constitutively active PI3K, NF-κB, and secretion of uPA, which is responsible for the migration of highly invasive breast cancer cells (Sliva *et al.*, 2002). In another study, constitutive activation of Akt was identified in breast cancer cells, while benign breast epithelial cell lines were immortalized through pathways that are independent of the EGF/PI3K/Akt kinase cascade, but this was not associated with invasiveness (Zhang *et al.*, 2003). Transfection of constitutively active Akt caused accelerated cell division and osteopontin expression (Zhang *et al.*, 2003). Conversely, dominant-negative Akt kinase slows cell cycle progression and suppresses osteopontin expression (Zhang *et al.*, 2003). The manipulation of osteopontin expression in this setting by transfection of the gene or its antisense did not affect the growth rate of the cells but altered cell motility and anchorage independence (Zhang *et al.*, 2003). Therefore, Akt kinase was postulated to activate two distinct genetic programs: the program of growth and survival and the program of invasiveness and anchorage independence, which is mediated by osteopontin (Zhang *et al.*, 2003). These studies define Akt kinase as a molecular bridge between cell cycle progression and dissemination. In colorectal cancer, another group investigated the effect of inhibiting the PI3K/Akt/IKKβ pathway in regulating the inappropriate constitutive activation of NF-κB and β-catenin (Agarwal *et al.*, 2005). Inducible expression of either dominant-negative IKKβ or PTEN strongly inhibited both the constitutive NF-κB- and β-catenin-dependent promoter and endogenous gene activation (Agarwal *et al.*, 2005). Targeted array-based gene expression analysis of this inducible system reveals that many of the genes downregulated upon inhibition of this pathway were involved in tumor angiogenesis and metastasis (Agarwal *et al.*, 2005).

In this report, we have systematically investigated the respective roles of the class I_A PI3K catalytic isoforms in the migration of breast epithelial cells and invasive (fibroblastoid) cells generated by transduction with oncogenic H-Ras and stimulation with TGF-β1 (Oft *et al.*, 1996). Using this system, we show for the first time that the PI3K isoforms p110α, p110β and p110δ have opposing roles in the migration of breast epithelial cells and transformed cells. The p110α, p110β isoforms were found to promote cell migration in invasive (fibroblastoid) cells, while the p110δ isoform restricts the migration of normal breast epithelial cells and is down-regulated during the process of epithelial to mesenchymal transition (EMT) induced by oncogenic H-Ras and TGF-β1.

MATERIALS AND METHODS

Cell Culture

EpH4 murine epithelial cells stably transfected with the H-Ras oncogene (EpRas) and induced to undergo EMT upon addition of TGF-β1 (FibRas) were previously described (Oft *et al.*, 1996). The three cell lines employed in this study were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS), 1% glutamine, 1% penicillin/streptomycin. In the case of EpRas and FibRas cells the culture medium was supplemented with G418 (0.5 mg/ml, Calbiochem).

Antibodies and Reagents

The following antibodies were used: p85α, p110α, p110β, p110δ, PTEN, Erk1/2, Akt/PKB, Cdc42, Integrin α5, Eps8 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated S6 protein (Ser240/244), phosphorylated Erk1/2, phosphorylated Akt (Ser473), Rac1 (Cell Signaling Technology, Danvers, MA), β-Tubulin, β-actin (Sigma-Aldrich, St. Louis,MO). The constructs encoding for p110α, p110β and p110δ as wild type and kinase dead were in pcDNA3 (Invitrogen) or pACCMV. The constructs encoding activated or kinase-inactive Akt were from Upstate. The activated S6K1 construct (T412D) or kinase-inactive S6K1 were in pcDNA3. The plasmids pcDNA3-EGFP-RhoA-T19N, pcDNA3-EGFP-RhoA-Q63L, pcDNA3-EGFP-RhoA-wt, pcDNA3-EGFP-cdc42-Q61L, pcDNA3-EGFP-cdc42-T17N, pcDNA3-EGFP-cdc42-wt, pcDNA3-EGFP-Rac1-wt, pcDNA3-EGFP-Rac1-T17N, pcDNA3-EGFP-Rac1-Q61L were obtained from the Addgene Plasmid Repository. The p110α shRNA and p110β shRNA were from Open Biosystems (Celbio). The isoform-specific PI3K inhibitor IC87114 was provided by ICOS Corporation (Indianapolis, IN, USA). LY294002, PD98059 and rapamycin were obtained from Calbiochem (La Jolla, CA).

SDS-PAGE and Western Blot Analysis

Cells plated on 10 cm-dishes were lysed in lysis buffer, and SDS-PAGE and Western blot was performed as previously described. The gels were transferred onto hydrophobic polyvinylidene difluoride membrane (Hybond-P; Amersham Bioscience). This membrane was than blotted with different antibodies. For detection, the enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences) were used according to the manufacturer's protocol.

Real-Time PCR Analysis

RNA was isolated using the RNeasy Mini Kit from Qiagen (Santa Cruz, CA). Reverse transcription polymerase chain reaction (RT-PCR) was then performed using the QIAGEN kit in agreement with the manufacturer's protocol. A real time PCR was then performed employing TaqMan (Applied Biosystems) primers and 18s rRNA control primers. For each sample 25 µl of reaction mix was used (100ng cDNA, 1µl each TaqMan primer, 12.5µl TaqMan Universal PCR Mix). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10min, and then 50 cycles at 95°C for 15s and 60°C for 1min. All the conditions were performed in triplicate and the relative expression of p110α, p110β and p110δ was calculated in accord with the C_T method.

Cell Migration, Wound Healing Assays

Cells were plated in 12-wells culture plates in complete culture medium in a way to be confluent after 24h. At that time a wound was created using a disposable 200 µl micropipette tip in order to scrape off the cells. Pictures were then taken every 30 min for 8 h using a Leica DM IRBE Inverse, wide field Microscope (EMZ, University of Zurich) in order to follow the wound closure. In the case of transfected cells the transfection was performed 48h in advance

and cells were plated 24h before the wound healing assay. For experiments with inhibitors, the compounds were added 40 min before the experiment and then replaced together with fresh medium.

Cell Motility, Time Lapse Assays

Cells were platted 24h before the experiment, in a 12-well plates in a way to be 30-40% confluent the day after. Cell migration was then monitored by taking pictures every 10 min for 12h. The program ImageJ was used to make a video. Single cells were followed and the speed of cells was measured under the different experimental conditions.

Time lapse imaging of random migration was performed on cells seeded at 30-40% confluence in a 12-well plate using a IX70 inverted microscope equipped with an incubator (Solent Scientific) for temperature an CO_2 control. Images were acquired every 10 min for 12 hours by a Sensys (Photometrics) CCD camera controlled by Metamorph Software (Universal Imaging) using a 10X magnification objective. Tracking of cells was performed using the "Manual Tracking" plugin distributed with ImageJ software. Data for velocity analysis were exported to Excel for further elaboration.

Cell Motility Assays

MDA-MB-231 cells (10^3 cells/well) were plated in optically-improved 96 well-plates (BD-Falcon). Oligonucleotide RNAi against Rac1, p110α, p110β, Akt1, Akt2, S6K1 or non-targeting sequences (Dharmacon) were transfected (35 nM final concentration) into the cells using the Dharmafect 2 transfection reagent (Dharmacon). Cells were then incubated at 37°C, 10% CO_2 for 48h to allow for target down-regulation prior to imaging by phase contrast using a motorised-staged environment-controlled Zeiss microscope. Alternatively, MDA-MB-231 cells were treated with the relevant inhibitors 1 hour prior to imaging. Time lapse acquisition (1 image/10 min/well for 18 hours) was achieved using a prior camera linked to the

Metamorph Software (Molecular Devices). Each condition was performed in quadruplicate. 15 cells were tracked per replicate (60 cells total) using Metamorph and tracks analysed by Mathematica 5 (Wolfram Research) to determine migration speed and persistence.

Cell Proliferation

Cells were plated at two different concentration $5x10^3$/well and $10x10^3$/well in 96-well plates and left to growth respectively 8h and 48h in complete DMEM in the presence or absence of inhibitors. For every condition 8 wells were plated. The number of viable cells was then measured by means of an MTS assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Transfections

Cells were transfected with cDNAs and shRNAs targeting p110α, p110β or p110δ using the Amaxa Nucleofector system (Amaxa biosystems, Gaithersburg, MD). Following the results from the Optimisation Kit, solution L and Program T-020 were chosen and used according to the manufacture's protocol.

Immunoprecipitations

Cells were lysed in RIPA buffer and then the protein concentration was measured by using the BCA protein assay (Pierce, Rockford, IL). The lysates was then incubated for 3 h with antibodies specific for Eps8 or p85α together with Protein G Sepharose beads (Amersham Biosciences). The immune complexes were then washed and denatured by boiling 5 min in SDS-PAGE buffer.

Rac/cdc42 Activity Assay

Glutatione *S*-transferase (GST)-PAK Cdc42 and Rac-binding region (CRIB) fusion protein was incubated with cell lysates in order to pull down GTP-bound Cdc42 and Rac as described previously (Katso *et al.*, 2006).

Rho Activity Assay

Cleared cell lysates were incubated with bacterially produced GST-Rho-binding domain of Rhotekin bound to glutathione-Sepharose beads (Sander *et al.*, 1999). The beads were washed twice with lysis buffer and bound proteins were eluted in SDS-sample buffer and analyzed by Western blotting with the use of anti-RhoA monoclonal antibody.

Immunofluorescence for F- actin

Cells were seeded on glass coverslips and let grown for 24 h. In the case of inhibitors, the cells were then treated for 3 days. Then cells fixed in methanol, and permeabilized in 0,1% Triton X-100 were incubated with rhodamine-conjugated phalloidin to detect F-actin. Nuclear staining was perfomed by incubating cells with DAPI before coverslip mounting. The coverslips were then washed in PBS and incubated in fresh Triton X-100 for 10-15 min in order to permeabilise the cells. After washing again in PBS1x an anti-actin antibody was added and after 20min was added also phalloidin and left for an additional 30 min. Coverslips were then washed in PBS, incubated with DAPI for 10 min and washed again PBS and finally fixed by Movieo on the cover slide.

RESULTS

Transformation of Mammary Epithelial Cells by Oncogenic H-Ras and TGF-β1 Induces Changes in the Expression of Class I_A PI3K Isoforms

To gain further insight into the contribution of class I_A PI3K isoforms in the acquisition of a metastatic phenotype by mammary epithelial cells, we initially compared the expression of components of the PI3K/Akt signalling pathway between normal (non-tumorigenic) EpH4 cells, EpRas (H-Ras-transduced and tumor forming) and FibRas (TGF-β1-transformed and tumor forming) (Oft *et al.*, 1996). This analysis revealed striking differences in the expression of class I_A PI3K catalytic isoforms. While the expression of p110α and p110β was increased in FibRas cells, as compared to EpH4 cells, the expression of p110δ was high in EpH4 cells, but undetectable in EpRas and FibRas cells, as assessed by Western blot analysis (Figure 1A). This change in p110δ expression paralleled a sharp decrease in the activation of Akt in EpRas and FibRas cells (Figure 1A). In contrast, activation of Erk1/2 and phosphorylation of the S6 protein were increased in EpRas and FibRas cells, as compared to EpH4 cells (Figure 1A). To further investigate the mechanism underlining the changes in expression class I_A PI3K catalytic isoforms, a quantitative RT-PCR analysis was performed. This revealed that p110α and p110β mRNA expression was higher in FibRas cells than in EpH4 cells, while p110δ mRNA expression was decreased in FibRas cells (Figure 1B). The migratory capacity of the three cell types was then compared in a wound-healing assay, which revealed an increase in the response in FibRas cells, as previously reported (Figure 1C). Moreover, FibRas cells also displayed an increased proliferation rate, as compared to EpH4 and EpRas cells (Figure 1D). Together these results revealed that the expression levels of class I_A PI3K p110α, β and δ are altered in an opposite manner during the process of transformation of EpH4 cells by oncogenic H-Ras and TGF-β1. Alterations were also observed in the activation status of Akt,

Erk1/2 and S6K, which may impact on the altered migratory and growth properties of FibRas cells, as compared to EphH4 cells.

The Class I_A PI3K Catalytic Isoforms Have Opposite Roles in the Migration of FibRas Cells

To investigate the contribution of the three class I_A PI3K catalytic isoforms in the migration of EpH4, EpRas and FibRas cells, the cells were transfected with constructs encoding wild type or kinase-inactive p110 subunits. The transfection efficiency using the optimised Nucleofection protocol was in the range of 80-90%, as assessed by FACS analysis of cells transfected with a reporter plasmid encoding green fluorescent protein (GFP) (data not shown). Transfection of EpH4 cells with wild type p110α increased the migration speed of the cells, while kinase-inactive p110α reduced the response in EpRas and FibRas cells (Figure 2A). In the case of p110β, transfection of EpH4 cells with the wild type enzyme moderately increased the migration speed of the cells, while the kinase-inactive mutant decreased the response in EpRas and FibRas cells, although the effect was much more prominent in FibRas cells (Figure 2C). Re-expression of wild type p110δ in EpRas and FiRas cells reduced the migration speed of the cells (Figure 2E), and the effect of the transfection could be inhibited by the p110δ inhibitor IC87114, indicating that the catalytic activity of the enzyme impairs migration in these cells (Figure 2E and data not shown). In contrast, transfection of kinase-inactive p110δ into EpH4 cells increased the migration speed of the cells (Figure 2E). Transfection of p110 constructs did not significantly affect the growth response of EpH4, EpRas or FibRas cells during the time range of the migration experiment (8 h) (Supplementary Figure S1, A, C and E). The role of three class I_A PI3K catalytic isoforms in the proliferation of EpH4, EpRas and FibRas cells was then investigated. Transfection of p110α wild type into EpH4 cells, or of kinase-inactive p110α into EpRas or FibRas cells did

not alter the proliferative response of the cells in a 48 h assay (Figure 2B). A partial decrease of EpH4 cell proliferation was observed upon transfection of wild type p110β (Figure 2D). FibRas cells transfected with wild type p110δ displayed a partial reduction of cell proliferation (Figure 2F). Together these results show that while p110α and p110β contribute to the migration of EpRas and FibRas cells, these isoforms do not appear to play an important role in cell proliferation. In contrast, p110δ expression appears to have a negative impact on the migration of EpRas and FibRas cells.

To confirm the results above, an RNAi approach targeting p110α and p110β was used. Vectors encoding short hairpin RNAs targeting p110α and p110β were transfected into EpH4, EpRas or FibRas cells and the impact on cell migration and proliferation was investigated as above. A Western blot analysis confirmed the selective down-regulation of p110α or p110β upon transfection with the specific shRNA constructs (Figure 3A). Down-regulation of p110α significantly impaired the migration of EpH4, EpRas and FibRas cells, although the effects of the shRNA were more pronounced in the two latter cell types (Figure 3B). In contrast the shRNA targeting p110β selectively impaired the migration of FibRas cells (Figure 3D). Surprisingly, p110β down-regulation by shRNA increased the migration speed of EpH4 cells (Figure 3D). The down-regulation of p110α or p110β by shRNA had only a limited effect on cell proliferation (48 h) of EpRas and FibRas cells (Figure 3, C and E), confirming the results obtained before (Figure 2, B and D). Transfection of shRNA constructs targeting p110α or p110β did not significantly affect the growth response of EpH4, EpRas or FibRas cells during the time range of the migration experiment (8 h) (Supplementary Figure S1, B and D).

To further validate the results obtained with transfections, the isoform-specific PI3K inhibitors YM024 (p110α), TGX-221 (p110β) and IC87114 (p110δ) were tested in migration and proliferation assays. The p110α inhibitor potently inhibited the migration of FibRas and EpRas cells (Figure 4A), while having only modest effects on cell proliferation (Figure 4B).

Similar results were obtained with the pharmacological p110β inhibitor, which impaired the migration of FibRas and to a lesser extent of EpRas cells (Figure 4C), while have no effect on cell proliferation (Figure 4D). The p110δ inhibitor produced an increase in the migration of EpH4 cells, while having no significant effect in EpRas or FibRas cells (Figure 4E). These results supported the findings obtained with kinase-inactive p110δ transfection into EpH4 cells (Figure 2E). IC87114 did not have a significant effect on the proliferation of the three cell types (Figure 4F). Treatment of the three cell types with the inhibitors targeting p110 isoforms resulted in only small effects on the growth response of EpH4, EpRas or FibRas cells during the time range of the migration experiment (8 h) (Supplementary Figure S2, D, E and F).

The mechanism of the p110δ-mediated inhibition of cell migration was subsequently investigated. Treatment of EpH4 cells with IC87114 resulted in a complete inhibition of Akt activation in these cells (Figure 4G). On the other hand, transfection of p110δ wild type into EpH4 or EpRas cells significantly enhanced Akt activation (Figure 4G). Together these data demonstrate that p110δ is a major contributor to Akt activation in EpH4 cells and that down-regulation of p110δ expression in EpRas and FibRas cells is probably the cause of the decrease in Akt activation observed in these cells (Figure 1A).

The effect of generic inhibitors of PI3K (LY294002), MEK/Erk (PD98059) and mTOR (rapamycin) were then tested on the migration and proliferation of EpH4, EpRas or FibRas cells. The PI3K inhibitor strongly impaired the migratory response of EpRas and FibRas cells, and to a lesser extent EpH4 cells (Figure 5A). The proliferation of all three cell types was affected to a similar extent by treatment with LY294002 (Figure 5B). In contrast, the MEK/Erk inhibitor failed to significantly affect the migration of either EpH4, EpRas, or FibRas cells (Figure 5C). However, it inhibited the proliferation of all three cell types (Figure 5D). Rapamycin inhibited the migration of EpH4 and FibRas cells, but not of EpRas cells

(Figure 5E), while it inhibited the proliferative response in all three cell lines (Figure 5F). Treatment of the three cell types with the inhibitors resulted in only small effects on the growth response of EpH4, EpRas or FibRas cells during the time range of the migration experiment (8 h) (Supplementary Figure S2, A, B and C). Together these data indicate that both the PI3K and the MEK/Erk pathways are required for cells proliferation EpH4, EpRas or FibRas cells, PI3K has a selective role in cell migration.

The PI3K Downstream Targets Akt, S6K and Rac Have Opposite Roles in the Migration of FibRas Cells

To further dissect the signalling pathways involved in the migration of EpH4, EpRas and FibRas cells, the involvement of the downstream targets of PI3K pathway were investigated. Transfection of constitutively activated Akt1 into EpH4, EpRas or FibRas cells reduced the migratory response of the cells, while an inactive mutant of Akt had no effect on the response (Figure 6A). In contrast, a catalytically inactive mutant of S6K reduced the migration of EpH4, EpRas and FibRas cells (Figure 6B).

The role of Rho/Rac family GTPases in the migratory response of EpH4, EpRas and FibRas cells was then studied. An activated mutant of Cdc42 selectively increased the migration of EpH4 cells, while dominant-negative Cdc42 impaired the response in EpRas and FibRas cells (Figure 6C). Comparable results were obtained with Rac, since activated Rac1 increased the migration of EpH4 cells, while the corresponding dominant-negative mutant inhibited the response (Figure 6D). In contrast, activated Rho inhibited the migration of EpRas and FibRas cells, while having no effect in EpH4 cells (Figure 6E). Dominant-negative Rho stimulated the migration of EpH4 cells, but had no effect in EpRas and FibRas cells (Figure 6E).

Transfection of the activated and dominant negative mutants of Akt, S6K, Cdc42, Rac and Rho did not significantly affect the growth response of EpH4, EpRas or FibRas cells during

the time range of the migration experiment (8 h) (Supplementary Figure S3, A-E). Together these results show that while Akt and Rho appear to have a negative regulatory role in the migration of FibRas cells, S6K, Rac and Cdc42 have a positive role in the response.

The ability of the class I_A PI3K isoforms to couple to the downstream targets Akt, S6K, Cdc42 and Rac was then investigated. The activity of Cdc42 and Rac was higher in FibRas cells, as compared to EpH4 and EpRas cells (Figure 7B). In contrast, no increase in the activity of Rho was observed in FibRas cells (Figure 7B). Inhibition of either p110α or p110β with pharmacological compounds impaired S6 protein phosphorylation in FibRas cells, while the p110δ inhibitor had no effect (Figure 7A). Moreover, the p110α inhibitor impaired the activation of Rac and Cdc42 in FibRas cells, while the p110β inhibitor selectively affected Cdc42 activation (Figure 7B).

The Catalytic Activity of the Class I_A PI3K p110α and p110β Is Required to Maintain the Migration Speed of FibRas and Human Breast Cancer Cells

To validate the results obtained in the wound-healing assay, the migration speed of FibRas cells was measured in a random-migration assay, using time-lapse microscopy. Transfection of kinase-inactive p110β into FibRas cells strongly reduced the migration speed of FibRas cells, while the effect of kinase-inactive p110α was less prominent (Figure 8A). Isoform-specific inhibitors of p110α or p110β strongly reduced the migration speed of FibRas cells, while the p110δ inhibitor had no effect on the response (Figure 8B).

In order to validate the findings obtained in FibRas cells, the impact of small interfering RNA (siRNA) or pharmacological inhibitors on the migration of the human breast cancer cell line MDA-MB-231 was studied. The siRNAs targeting Rac1, p110β and S6K1 significantly reduced the migration speed of the cells (Figure 8C). The siRNA targeting p110α or Akt1 had not significant effect on the migration of MDA-MB-231 cells, while the

Akt2 siRNA inhibited the response. However, under the conditions of the assay the Akt2 siRNA also induced cell death in MDA-MB-231, indicating that its effect on the migratory response is indirect. Moreover, the generic inhibitors LY294002, rapamycin and the isoform-specific inhibitors TGX-221 and YM024 significantly reduced the migration speed of MDA-MB-231 cells (Figure 8D).

The Class I_A PI3K p110α and p110β Isoforms Play an Important Role in the Cytoskeletal Organization of FibRas Cells

In order to investigate whether class I_A PI3K isoforms are involved in the cytoskeletal organization of EpH4 and FibRas cells, the effect of isoform-specific PI3K inhibitors on the actin cytoskeleton was investigated. EpH4 cells displayed few filopodia, no lammelipodia or ruffles, but abundant stress fibres (Figure 9A). Treatment of EpH4 cells with IC87114 induced the formation of some filopodia and lamellipodia, and the disappearance of stress fibres (Figure 9B). In contrast to EpH4 cells, FibRas cells displayed numerous filopodia and lamellipodia, but no stress fibres (Figure 9C). Treatment of these cells with the p110α inhibitor induced the disappearance of filopodia and lamellipodia (Figure 9D). Similar results were obtained with the p110β inhibitor TGX-221 (Figure 9E). Together these data show that the p110α and p110β isoforms play an important role in the cytoskeletal organisation of FibRas cells, in particular in the formation of filopodia and lamellipodia. In contrast, p110δ appears to play a negative role in the development of these structures in EpH4 cells, confirming the results obtained in the migration assays (Figure 4E).

Eps8 Interacts with Class I_A PI3K and Is Involved in the Migration of FibRas Cells

Previous work has shown that the Eps8/Abi1/Sos-1 complex plays an important role in Rac activation downstream of class I_A PI3K (Innocenti *et al.*, 2003). Therefore the involvement of Eps8 in the migration of FibRas cells was investigated. Co-immunoprecipitation experiments

revealed a constitutive interaction between Eps8 and the regulatory p85α subunit of class I_A PI3K in FibRas cells (Figure 10A). FibRas cells were then transfected with an Eps8 which is unable to bind Abi1 (Eps8 ΔSH3). The mutant Eps8 construct significantly impaired the migration of FibRas cells (Figure 10B), confirming the importance of the Eps8-Abi1-Sos-1 complex in the response.

DISCUSSION

The role of PI3K signalling in cell migration is well documented and previous reports have investigated the role of the catalytic class I_A PI3K isoforms in the migratory responses of various cells, including macrophages, neutrophils and fibroblasts (Barber and Welch, 2006). In breast cancer, an initial report described the role of p110β and p110δ in the migration of human breast cancer cell lines, while p110α was not involved in the response (Sawyer *et al.*, 2003). In addition there exists some controversy about the role of the downstream targets of PI3K such as Akt in the migration of breast cancer cells (Yoeli-Lerner *et al.*, 2005; Yoeli-Lerner and Toker, 2006).

In the present report, we have systematically compared three different breast cell types (normal, epithelial Ras-transformed and fibroblastoid Ras-transformed) for their requirement for PI3K signalling and catalytic class I_A PI3K isoforms in cell migration and proliferation. PI3K signalling and class I_A PI3K isoforms, did not play a substantial role in the induction of EMT by oncogenic H-Ras and TGFβ1, which supports previous studies using H-Ras effector mutants (Janda *et al.*, 2002). In addition, although the generic inhibitors of the PI3K signalling pathway LY294002 and rapamycin inhibited the proliferation of FibRas cells, inhibition of class I_A PI3K isoforms individually, did not affect the response to a significant extent. This observation could be interpreted by a certain degree of redundancy between individual class I_A PI3K isoforms for cell proliferation in this system, or by the contribution of other classes of PI3K isoforms (class I_B, II and III). However, inhibition of PI3K dramatically impaired the migration of FibRas cells, while inhibition of the MEK/Erk pathway did not significantly affect the response, suggesting a selective requirement for PI3K signalling in the migratory response. In contrast, MEK/Erk signalling was essential for cell proliferation of EpRas and FibRas cells, indicating that the PI3K and MEK/Erk pathways have divergent roles in the control of cell proliferation and migration in this cell system.

MEK/Erk signalling was also previously reported to be essential for the induction of EMT, while PI3K signalling did not make a substantial contribution to the response (Janda *et al.*, 2002).

During the process of EMT however, transfection of EpH4 cells with oncogenic H-Ras and stimulation with TGFβ1 led to a switch in the class I_A catalytic subunits, p110δ being down-regulated, while the expression of p110α and p110β was increased. This change in PI3K isoform expression occurred predominantly at the transcriptional level for p110α and p110β, while p110δ mRNA expression was reduced only in FibRas cells, although expression of p110δ protein was low in EpRas and FibRas cells. This suggests a contribution of post-transcriptional control mechanisms in the regulation of p110δ expression in this system. The PI3K isoform switch accompanied the increase in the migratory capacity of FibRas cells, as compared to EpH4 and EpRas cells, implying that the class I_A catalytic isoforms may differentially regulate cell migration in this system. This model was supported by the observation that p110δ inhibition increased the migratory capacity of EpH4 cells, and that re-expression of this isoform in EpRas and FibRas cells impaired the response. p110β appeared to play a major role in cell migration of FibRas cells, which paralleled its increased expression in this cell type. Concerning p110α, less selectivity was observed, since inhibition of this isoform impaired migration in all three cell types, to a similar extent.

In parallel to the induction of EMT and the p110 isoform switch, activation of Akt was decreased in EpRas and FibRas cells, while the activation of S6K and Erk was augmented. It can thus be speculated that Akt and S6K couple to distinct PI3K isoforms in this system, Akt being more dependent on p110δ, while S6K activation is controlled by p110α and p110β. This model is supported by the observation that p110δ inhibition decreased Akt activation in EpH4 cells, while re-expression of this isoform in EpRas cells restored Akt activation. Furthermore, S6K activation in FibRas cells was impaired by p110α and p110β inhibitors.

The inhibitory role of Akt in cellular migration described here supports previous findings in breast cancer cells (Yoeli-Lerner *et al.*, 2005).

In addition to Akt and S6K, Rho/Rac family GTPases appeared to differentially control the migration of EpH4/EpRas/FibRas cells. Inhibition of Cdc42 and Rac impaired the migration of EpRas and FibRas cells, and an activated mutant of Cdc42 stimulated the migration of EpH4 cells. Moreover, Cdc42 and Rac activity was enhanced in FibRas cells, as compared to EpH4 cells. In contrast, inhibition of Rho stimulated the migration of EpH4 cells, while an activated mutant of Rho impaired the migration of EpRas and FibRas cells. In FibRas cells, p110α activity was required to support Cdc42 and Rac activity, while p110β contributed to Cdc42 activation. Thus, increased p110α and p110β expression levels in FibRas cells most likely stimulate Cdc42/Rac activity, leading to enhanced cell migration.

Rho/Rac family GTPases have previously been shown to control the formation of different actin structures involved in the regulation of cell migration and adhesion (Hall, 1998; Bustelo *et al.*, 2007). EpH4 cells displayed a greater number of stress fibers, as compared to FibRas cells, while the latter cells displayed enhanced formation of filopodia and lamelipodia/ruffles, consistent with a migratory phenotype. Inhibition of p110α and p110β activity impaired the formation of filopodia and lamelipodia/ruffles in FibRas cells, which is consistent with an inhibition of cell migration. In contrast, p110δ inhibition in EpH4 cells lead to a disassembly of stress fibers and an increase in filopodia and lamelipodia/ruffle formation, consistent with enhanced cell migration. In view of the involvement of Cdc42 in the formation of filopodia and Rac in lamelipodia/ruffle formation, it can be hypothesized that increased p110α and p110β expression levels in FibRas cells stimulate Cdc42/Rac activity, leading to filopodia and lamelipodia/ruffle formation, which are involved in the process of cell migration. The present report also identifies the GTP exchange factor Eps8 as a possible contributor to Rac activation in FibRas cells downstream of class I_A PI3Ks. Indeed, an interaction between Eps8 and the regulatory p85α subunit of class I_A PI3K could be

documented in FibRas cells, and a dominant negative Eps8 mutant partially inhibited the migratory response of FibRas cells. It is presently still unclear whether the inhibition of p110δ/Akt activity in EpH4 cells leads to a disassembly of stress fibers via an effect on Rho, or whether p110δ/Akt activity negatively regulates the formation of filopodia and lamelipodia/ruffles in these cells.

Together our data provide novel information about the molecular mechanisms involved in the acquisition of a malignant invasive phenotype by breast epithelial cells in response to oncogenic H-Ras transduction and TGFβ1. The ability of these stimuli to promote a migratory phenotype appears to involve the activation of pro-migratory signalling pathways (S6K, Cdc42/Rac) and the inhibition of anti-migratory pathways (Akt) through modulation of the expression of the catalytic class I_A PI3K isoforms.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. The expression levels of class I_A PI3K isoforms are altered during the acquisition of a malignant metastatic phenotype in mammary epithelial cells. (A) Western blot analysis of equal amounts of cell lysate from EpH4, EpRas and FibRas cells with antibodies specific for the proteins indicated. (B) Quantitative RT-PCR analysis (TaqMan) for p110α, p110β and p110δ in the cell lines under study. (C) The migration speed of the cell lines was measured by using the wound healing assay. (D) Cell proliferation was measured by using a MTS assay. (Student's *t* test **p<0.01).

Figure 2. The catalytic class I_A PI3K isoforms play divergent roles in cell migration. (A-F) EpH4, EpRas and FibRas were transfected with plasmids encoding p110α, p110β or p110δ as either wild type (wt) or dominant negative (DN). Cell migration was measured 48 h after transfection using the wound healing assay (A, C, E). Cell proliferation was measured after 72 h using the MTS assay (B, D, F). (Student's *t* test **p<0.01).

Figure 3. The class I_A PI3K p110α and p110β play a role in the migration in FibRas cells. (A) EpH4, EpRas and FibRas where transfected with shRNA targeting p110α or p110β (two different sequences C12 and G11) or with the vector containing a non-targeting sequence (v). The cells were lysed 24h after transfection and a Western blot analysis was performed to confirm target down-regulation. (B, D) Cell migration was measured 48 h after transfection using the wound healing assay. (C, E) Cell proliferation was measured after 72 h using the MTS assay. (Student's *t* test **p<0.01).

Figure 4. Different isoforms of class I_A PI3K play specific roles in migration of FibRas cells. (A-F) EpH4, EpRas or FibRas cells were incubated with increasing concentrations of the

p110α inhibitor YM024 (A and B), the p110β inhibitor TGX-221 (C and D) or the p110δ inhibitor IC87114 (E and F). Cell migration was measured using the wound healing assay (A, C, E). Cell proliferation was measured after 72 h using the MTS assay (B, D, F). (G) EpH4 (left panel) or EpRas cells (right panel) were transfected with p110δ wild type or treated with the inhibitor IC87714 (1 μM). A Western blot analysis was performed to confirm p110δ expression (myc tag) and assess Akt activation (Ser473 phosphorylation). (Student's *t* test **p<0.01).

Figure 5. The PI3K pathway plays a major role in cell migration, while the MEK/Erk pathway is dispensable. (A-F) EpH4, EpRas or FibRas cells were incubated with increasing concentrations of the PI3K inhibitor LY294002 (A and B), the MEK inhibitor PD98059 (C and D) or the mTOR inhibitor rapamycin (E and F). Cell migration was measured using the wound healing assay (A, C, E). Cell proliferation was measured after 72 h using the MTS assay (B, D, F). (Student's *t* test **p<0.01).

Figure 6. The PI3K downstream targets Akt and S6K, as well as Rho family GTPases contribute to the migration response of breast cancer cells. (A and B) EpH4, EpRas and FibRas were transfected with plasmids encoding Akt1 or S6K1 as either activated mutant (CA) or dominant negative (KD). Cell migration was measured 48 h after transfection using the wound healing assay. (C-E). EpH4, EpRas and FibRas where transfected with plasmids encoding Cdc42 (C) or Rac (D) as either activated mutant (Q61L) or dominant negative (T17N), or Rho as either activated mutant (Q63L) or dominant negative (T19N). Cell migration was measured 48 h after transfection using the wound healing assay. (Student's *t* test *p<0.05, **p<0.01).

Figure 7. The class I_A PI3K p110α and p110β play specific roles in the activation of S6K, Cdc42 and Rac. (A) FibRas cells were incubated with the p110α inhibitor YM024 (1 μM), the p110β inhibitor TGX-221 (1 μM), the p110δ inhibitor IC87114 (1 μM), LY294002 (10 μM) or PD98059 (25 μM). A Western blot analysis was performed to investigate the status of S6 protein phosphorylation (Ser240/244). (B) EpH4, EpRAs or FibRas cells, in the presence or absence of YM024 (1 μM) or TGX-221 (1 μM) were lysed and GTP-bound Cdc42 and Rac purified using immobilised GST-PAK CRIB fusion protein. GTP-bound Rho was purified using immobilised GST-Rhotekin Rho-binding domain. The samples were analysed by Western blot using antibodies specific for the proteins indicated.

Figure 8. The class I_A PI3K p110α and p110β play a role in random migration of FibRas cells. (A) FibRas cells were transfected with p110α or p110β either as wild type (WT) or dominant negative (DN). Single cells were followed and the migration speed of cells was measured by time lapse assays. (B) FibRas cells were incubated with the p110α inhibitor YM024 (1 μM), the p110β inhibitor TGX-221 (1 μM), the p110δ inhibitor IC87114 (1 μM)and the migration speed of the cells measured as in (A). (C and D) The human breast cancer cell line MDA-MB-231 was transfected with siRNA targeting the proteins indicated or scrambled siRNA (sc) (A) or treated with vehicle (DMSO), IC87114, LY294002, TGX-221, YM024 (10 μM (high) or 1 μM (low)) or rapamycin (100 nM (high) 10 nM (low)). Time lapse acquisition was used to determine the migration speed. (Student's *t* test *$p<0.05$, **$p<0.01$, ***$p<0.001$).

Figure 9. The class I_A PI3K isoforms control different actin structures in EpH4 and FibRas cells. (A and B) EpH4 cells were treated with vehicle (A) or IC87114 (1 μM) (B). F-actin was visualised by fluorescent phalloidin staining. EpH4 cells present stress fibers (SF) and

filopodia (F) but in the presence of the inhibitor stress fibers disappeared and filopodia (F), and lamellipodia/ruffles (LR) appeared. (C-E) FibRas cells were treated with vehicle (C) YM024 (1 μM) or TGX-221 (1 μM). F-actin was visualised by fluorescent phalloidin staining. FibRas cells present lamellipodia/ruffles (LR) and filopodia, but no stress fibers (SF) and in the presence of the inhibitors these structures disappeared.

Figure 10. Interaction between Eps8 and p85α in FibRas cells. (A) FibRas cells were lysed and then an immunoprecipitaion for Eps8 and p85 was performed. The samples were analysed by Western blot for the proteins indicated. (B) FibRas cells were transfected with vector or an Eps8 mutant (ΔSH3). Cell migration was measured after 48 h using the wound healing assay. (Student's *t* test **$p<0.01$).

Figure 1

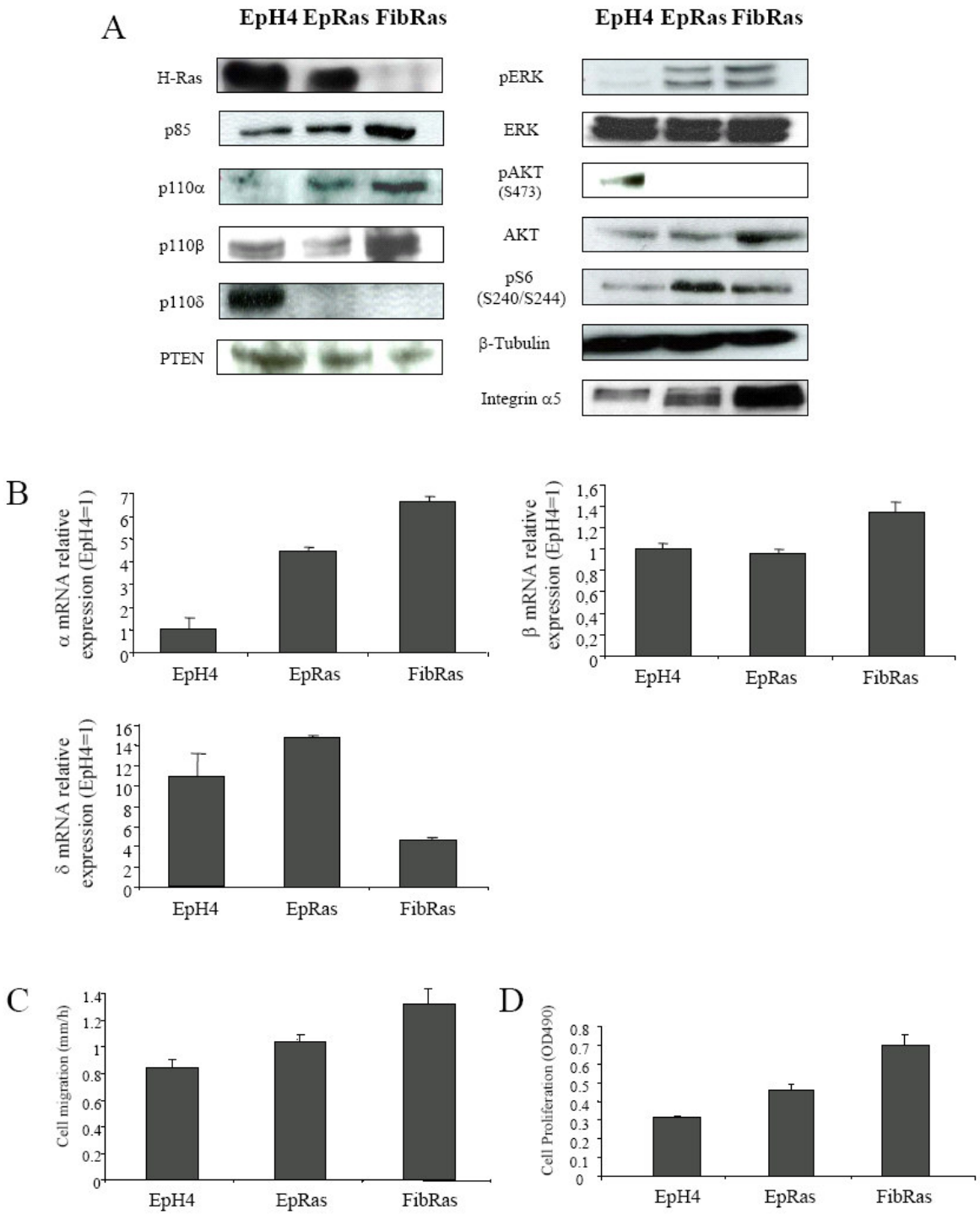

A
EpH4 EpRas FibRas
H-Ras
p85
p110α
p110β
p110δ
PTEN
EpH4 EpRas FibRas
pERK
ERK
pAKT (S473)
AKT
pS6 (S240/S244)
β-Tubulin
Integrin α5
B
α mRNA relative expression (EpH4=1)
EpH4
EpRas
FibRas
β mRNA relative expression (EpH4=1)
δ mRNA relative expression (EpH4=1)
C
Cell migration (mm/h)
D
Cell Proliferation (OD490)

Figure 2

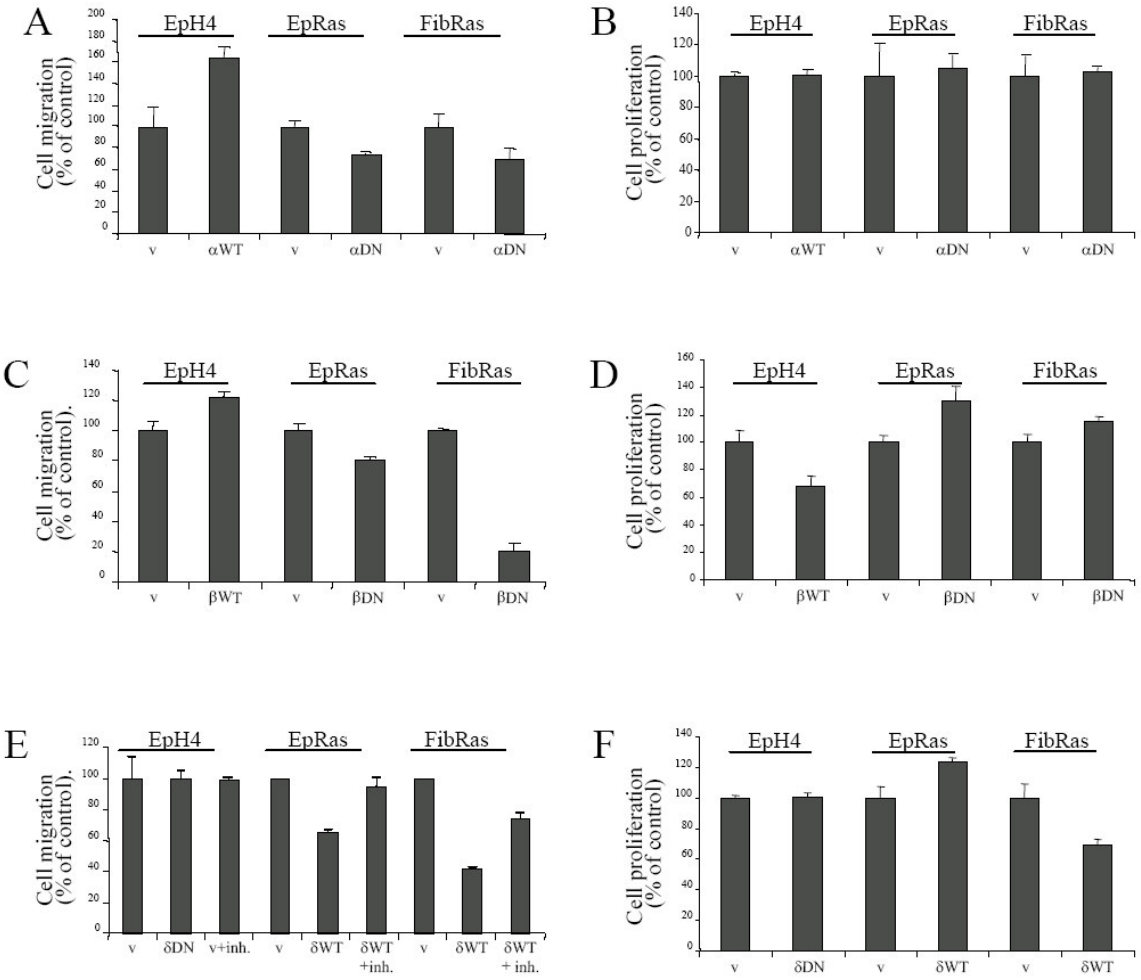

A
EpH4
EpRas
FibRas
Cell migration (% of control)
v
αWT
αDN
B
Cell proliferation (% of control)
C
Cell migration (% of control).
βWT
βDN
D
E
δDN
v+inh.
δWT
δWT +inh.
δWT + inh.
F

Figure 3

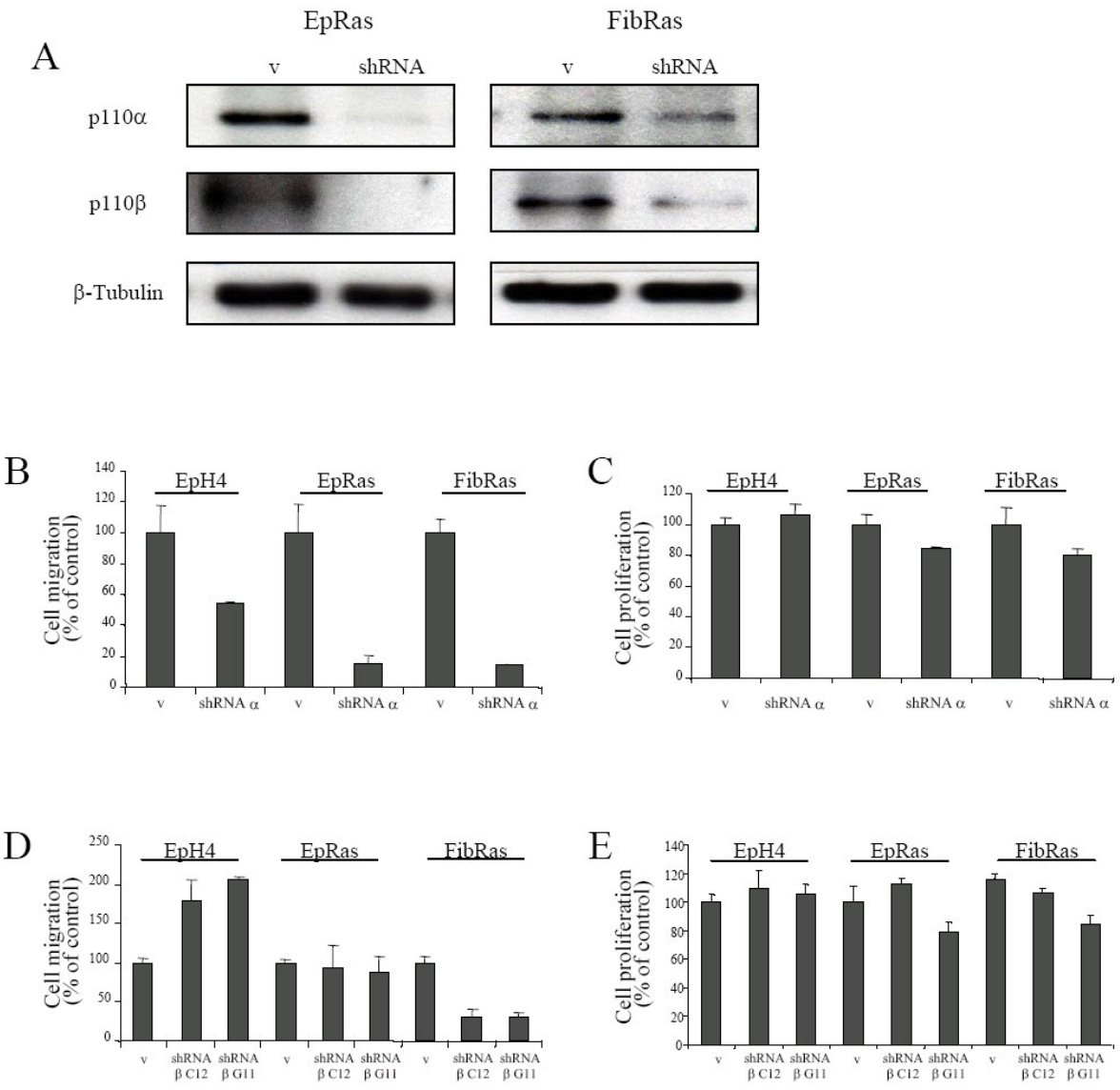

A
EpRas
FibRas
v
shRNA
v
shRNA
p110α
p110β
β-Tubulin
B
EpH4
EpRas
FibRas
Cell migration (% of control)
v
shRNA α
C
Cell proliferation (% of control)
D
shRNA β C12
shRNA β G11
E

Figure 4

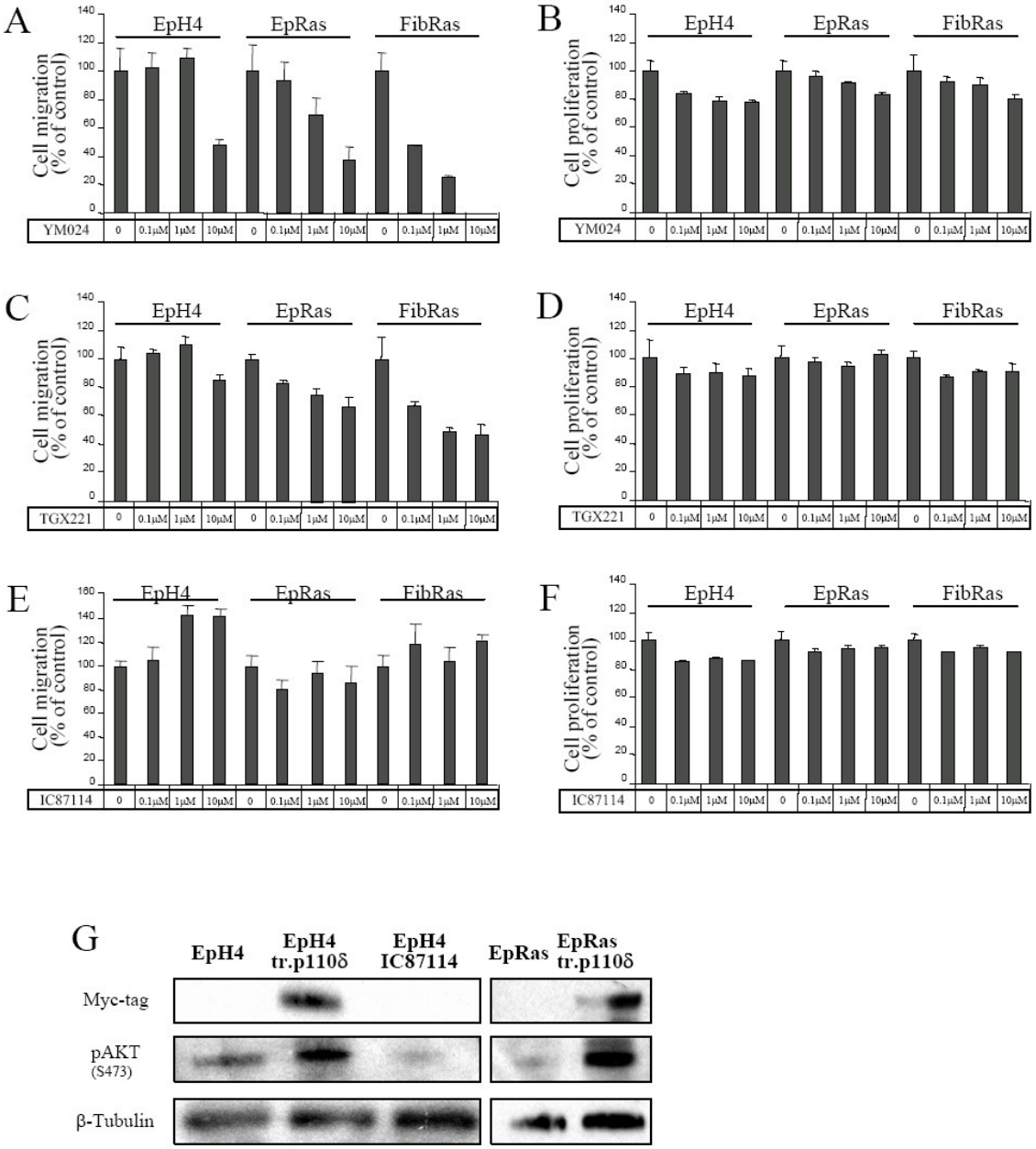

A
B
C
D
E
F
EpH4
EpRas
FibRas
Cell migration (% of control)
Cell proliferation (% of control)
YM024
TGX221
IC87114
0
0.1μM
1μM
10μM
G
EpH4
EpH4 tr.p110δ
EpH4 IC87114
EpRas
EpRas tr.p110δ
Myc-tag
pAKT (S473)
β-Tubulin

Figure 5

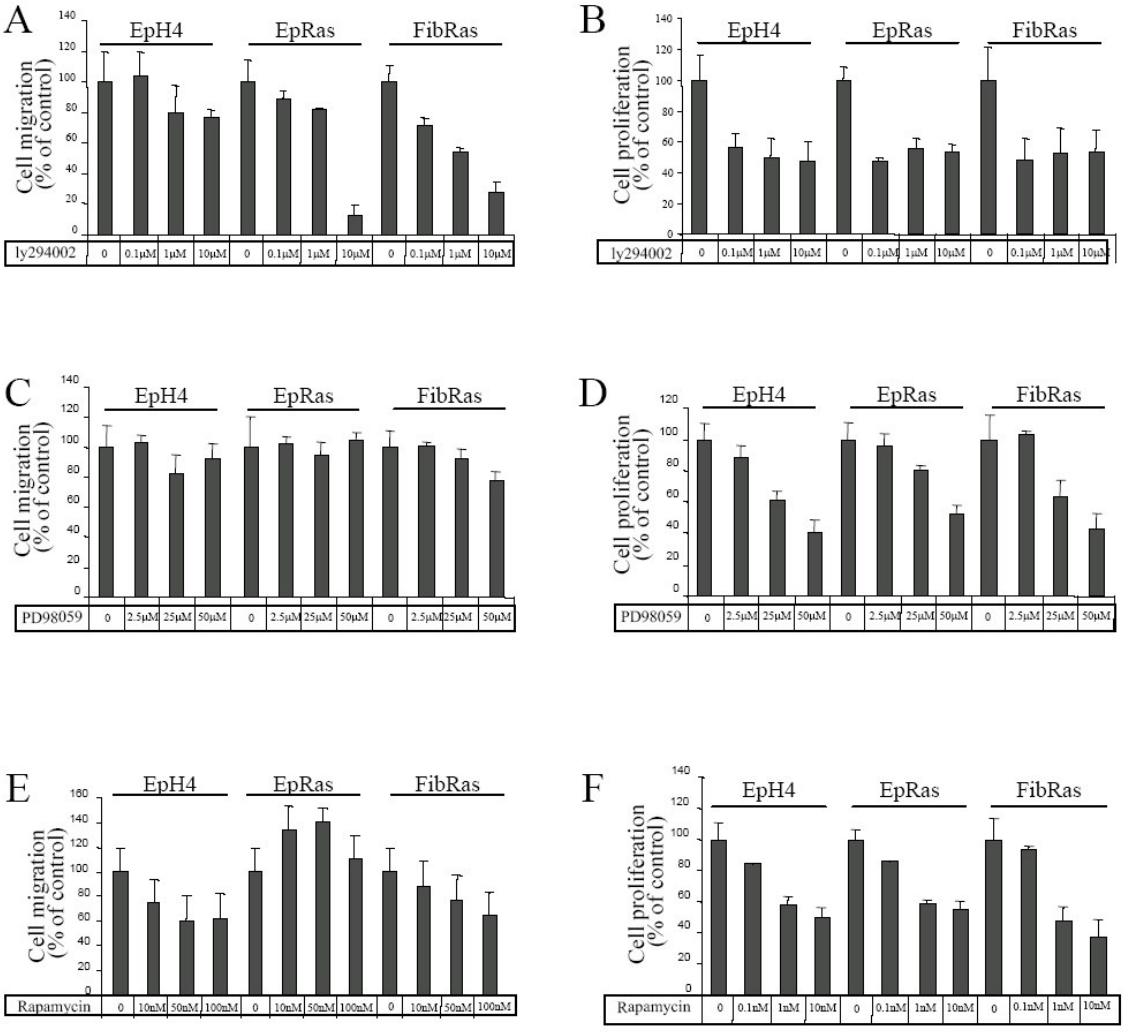

A
EpH4
EpRas
FibRas
Cell migration (% of control)
ly294002
0
0.1μM
1μM
10μM
B
Cell proliferation (% of control)
C
PD98059
2.5μM
25μM
50μM
D
E
Rapamycin
10nM
50nM
100nM
F
0.1nM
1nM

Figure 6

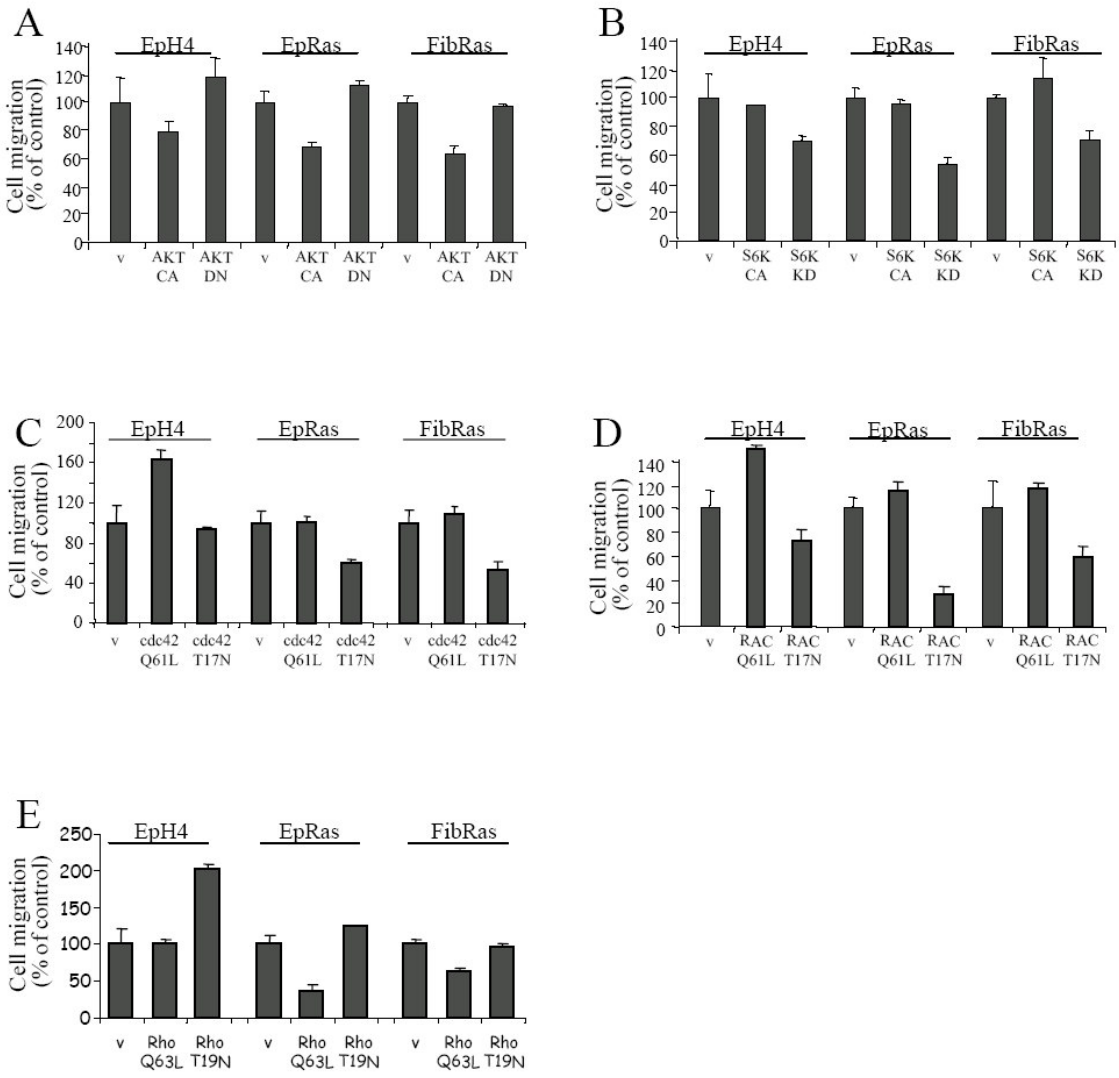

A
EpH4
EpRas
FibRas
Cell migration (% of control)
v
AKT CA
AKT DN
B
S6K CA
S6K KD
C
cdc42 Q61L
cdc42 T17N
D
RAC Q61L
RAC T17N
E
Rho Q63L
Rho T19N

Figure 7

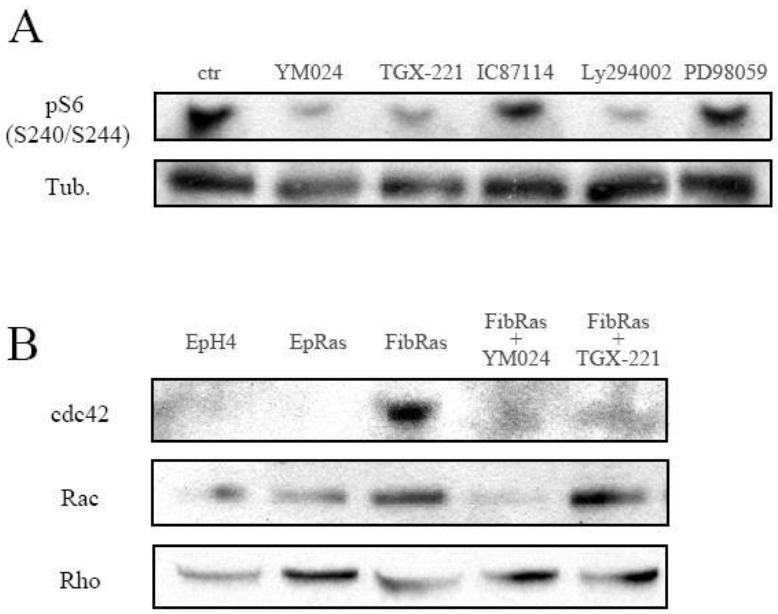
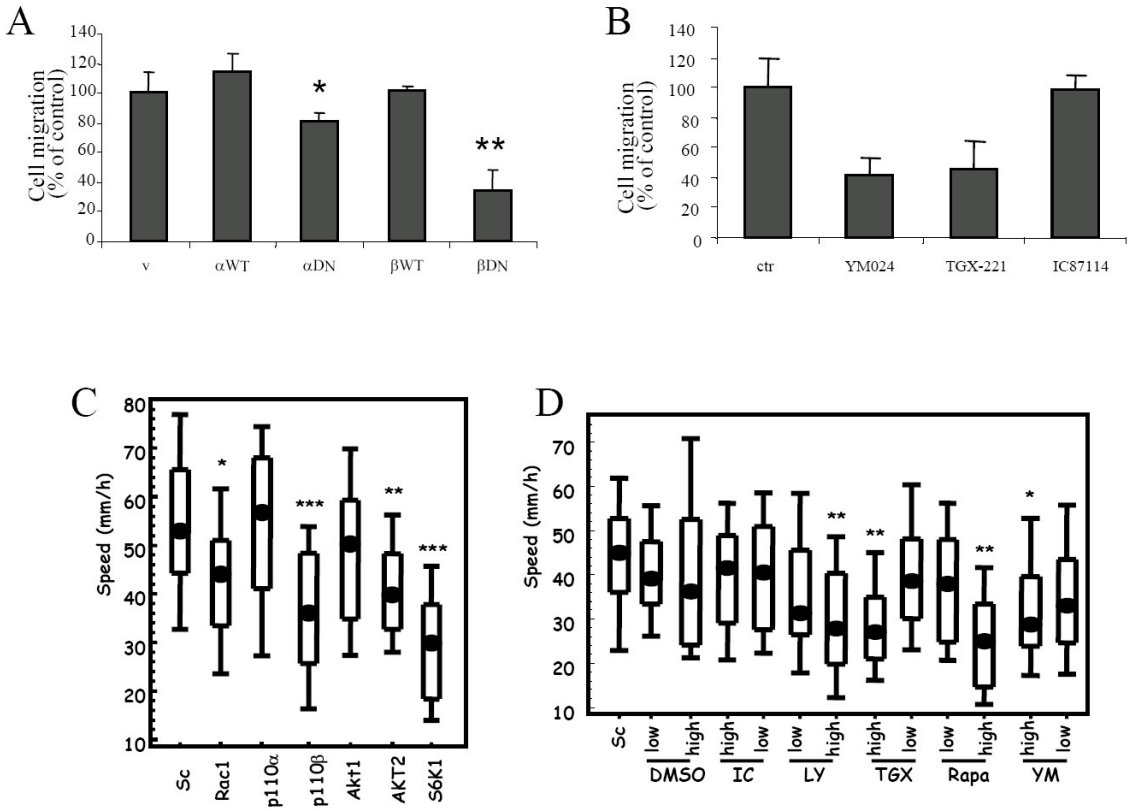

A
ctr
YM024
TGX-221
IC87114
Ly294002
PD98059
pS6
(S240/S244)
Tub.
B
EpH4
EpRas
FibRas
FibRas + YM024
FibRas + TGX-221
cdc42
Rac
Rho

Figure 8


A
Cell migration (% of control)
140
120
100
80
60
40
20
0
*
**
v
αWT
αDN
βWT
βDN
B
Cell migration (% of control)
ctr
YM024
TGX-221
IC87114
C
Speed (mm/h)
Sc
Rac1
p110α
p110β
Akt1
AKT2
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D
Speed (mm/h)
Sc
low
high
DMSO
IC
LY
TGX
Rapa
YM

Figure 9

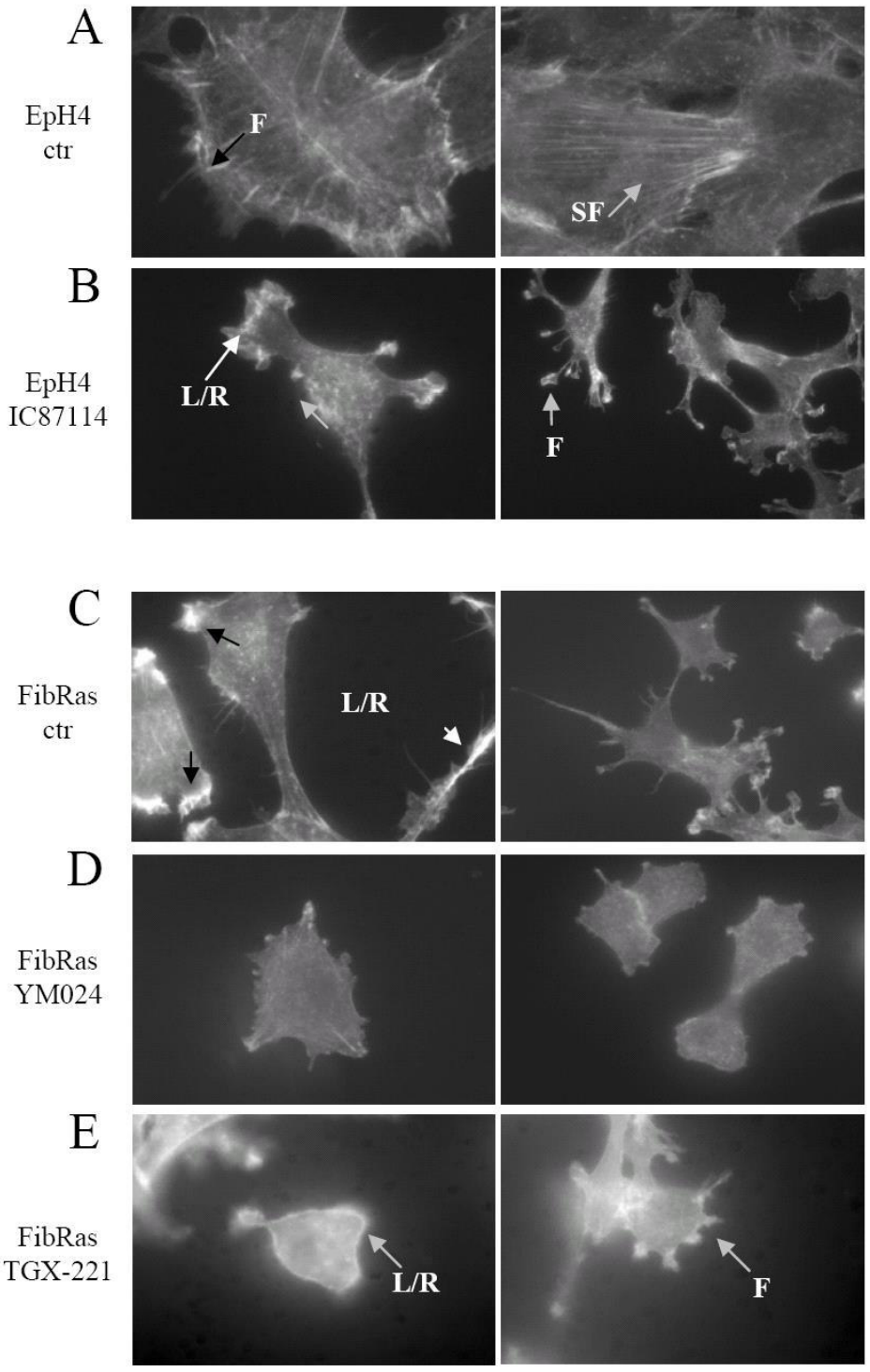
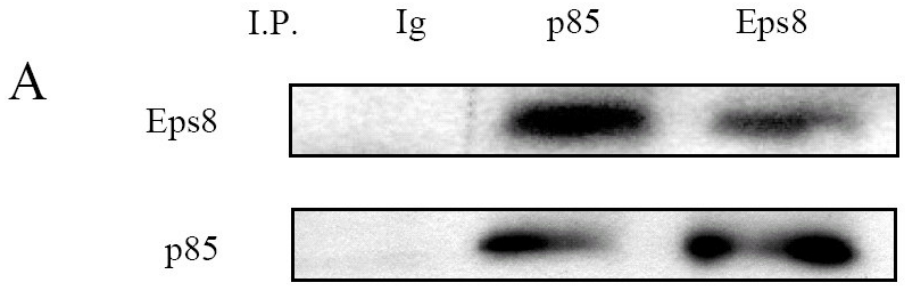

A
EpH4
ctr
F
SF
B
EpH4
IC87114
L/R
F
C
FibRas
ctr
L/R
D
FibRas
YM024
E
FibRas
TGX-221
L/R
F

Figure 10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I.P.
Ig
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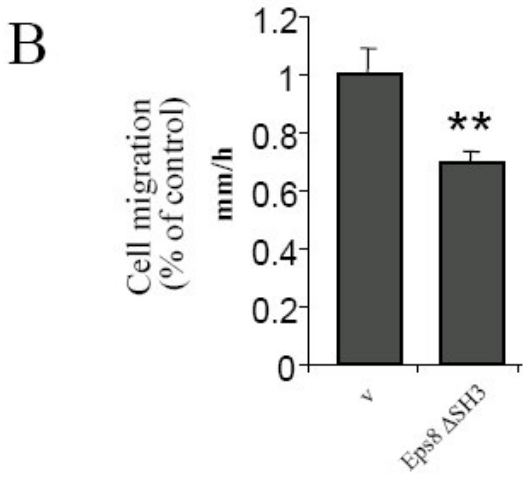
B
Cell migration
(% of control)
mm/h
1.2
1
0.8
0.6
0.4
0.2
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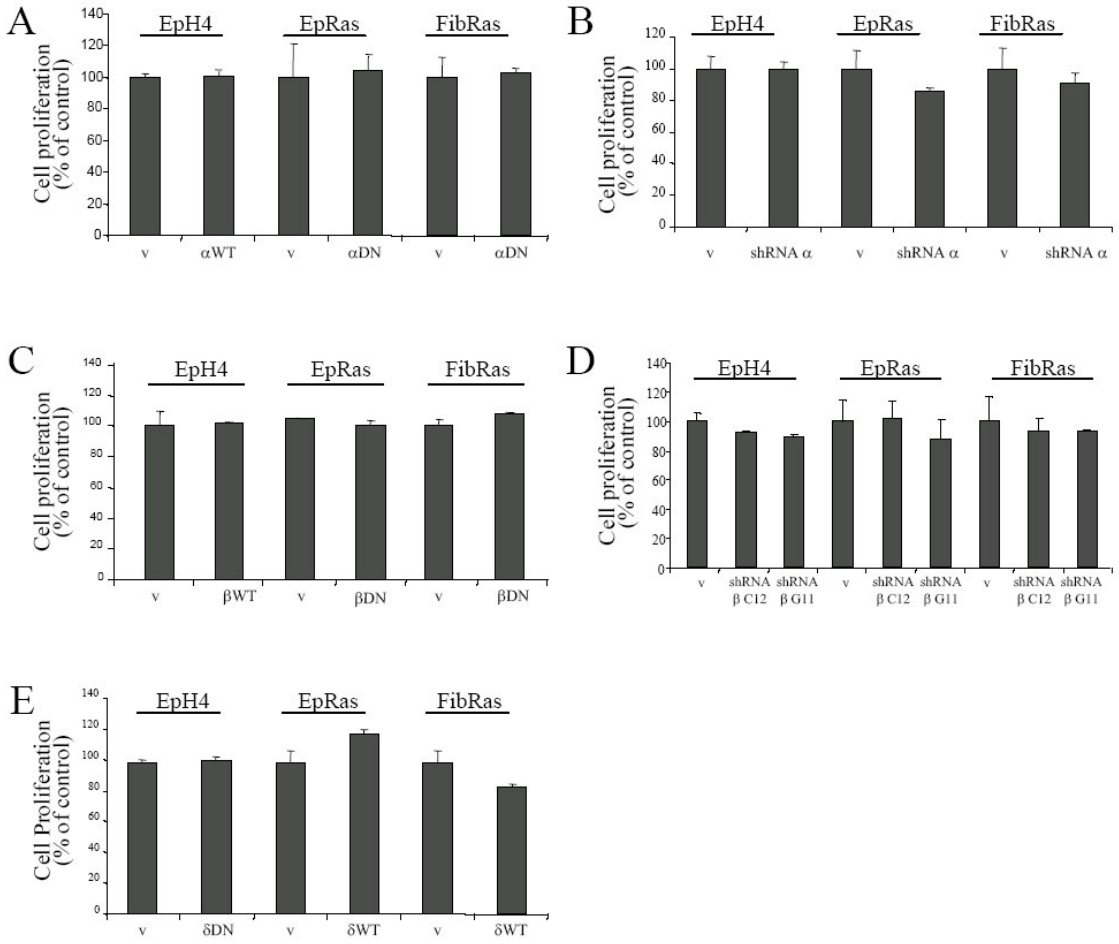
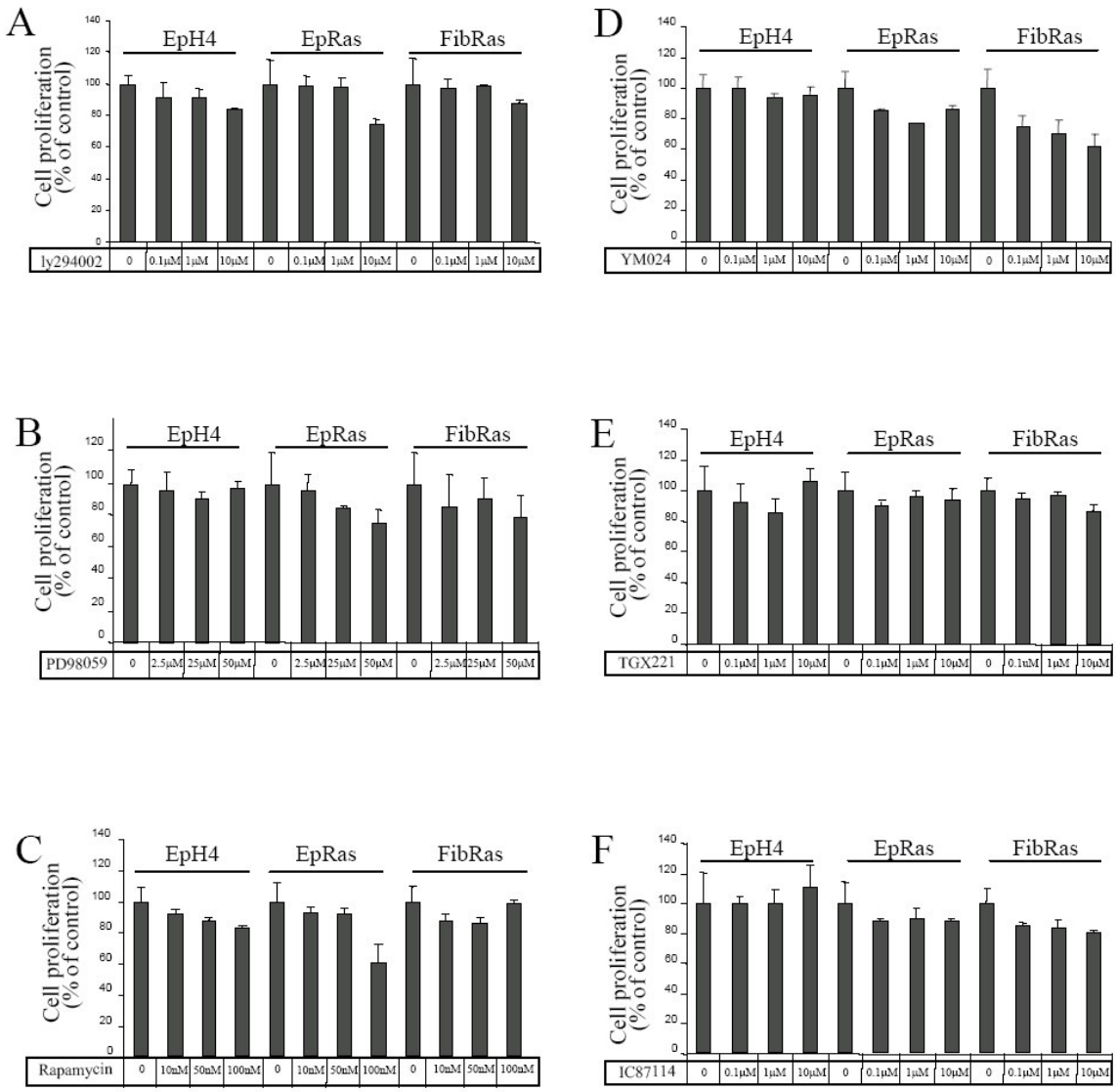
**
v
Eps8 ΔSH3

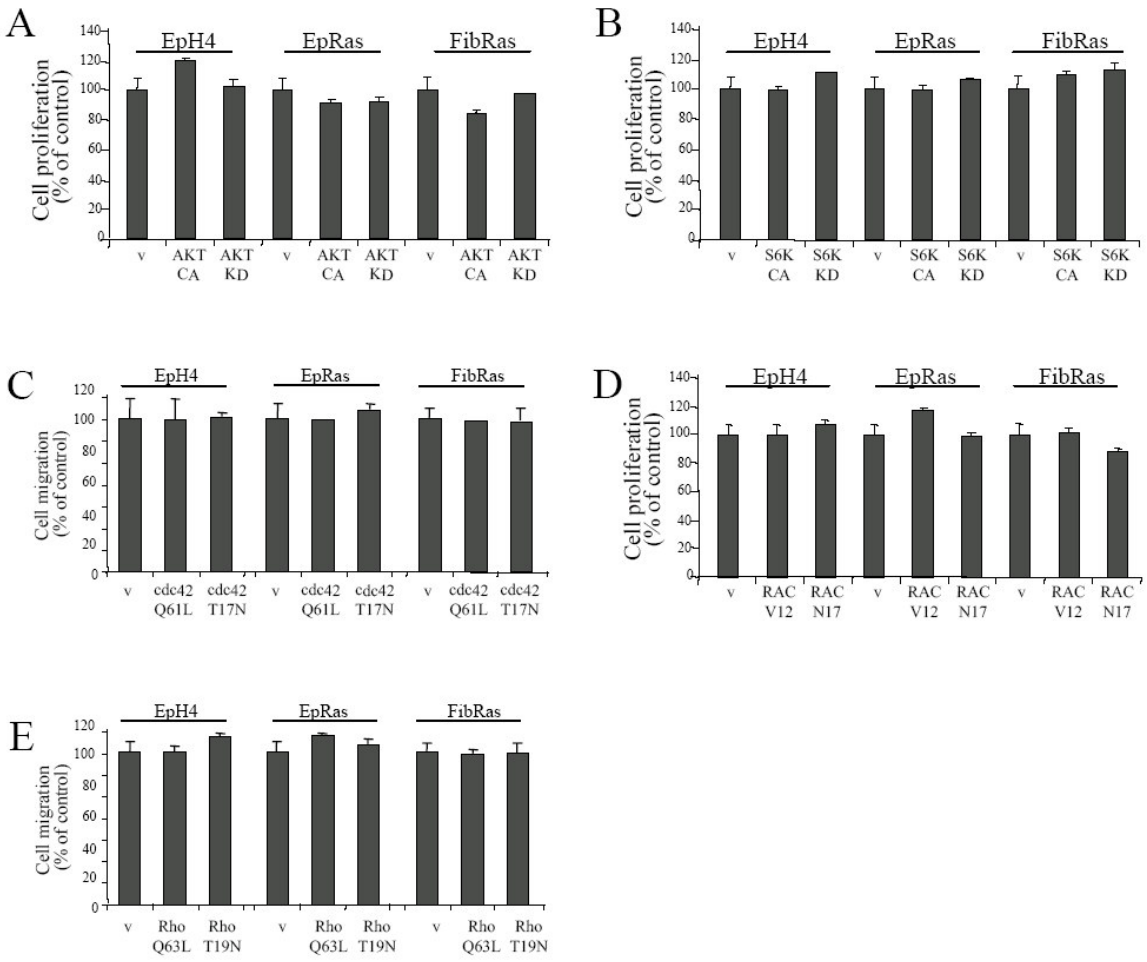
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



3.2.2 Phosphoinositide 3-kinase C2□ Regulares Cytoskeletal Organization and Cell Migration via Rac-dependent Mechanisms

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Phosphoinositide 3-Kinase C2β Regulates Cytoskeletal Organization and Cell Migration via Rac-dependent Mechanisms[D] [V]

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Receptor-linked class I phosphoinositide 3-kinases (PI3Ks) induce assembly of signal transduction complexes through protein–protein and protein–lipid interactions that mediate cell proliferation, survival, and migration. Although class II PI3Ks have the potential to make the same phosphoinositides as class I PI3Ks, their precise cellular role is currently unclear. In this report, we demonstrate that class II phosphoinositide 3-kinase C2β (PI3KC2β) associates with the Eps8/Abi1/Sos1 complex and is recruited to the EGF receptor as part of a multiprotein signaling complex also involving Shc and Grb2. Increased expression of PI3KC2β stimulated Rac activity in A-431 epidermoid carcinoma cells, resulting in enhanced membrane ruffling and migration speed of the cells. Conversely, expression of dominant negative PI3KC2β reduced Rac activity, membrane ruffling, and cell migration. Moreover, PI3KC2β-overexpressing cells were protected from anoikis and displayed enhanced proliferation, independently of Rac function. Taken together, these findings suggest that PI3KC2β regulates the migration and survival of human tumor cells by distinct molecular mechanisms.

INTRODUCTION

Phosphoinositide 3-kinases (PI3Ks) trigger activation of signaling pathways in response to extracellular stimuli that regulate diverse cellular programs such as cell survival, proliferation and migration, phagocytosis, and glucose homeostasis (Katso *et al.*, 2001). There are three classes of PI3Ks in mammals. Class I PI3Ks primarily generate PI(3,4,5)P_3, which binds to pleckstrin homology (PH) domains and activates a variety of proteins including the serine/threonine kinase PKB/Akt and exchange factors for small GTPases. Class II PI3Ks have the potential to generate PI(3,4,5)P3, but the type of PIPs that they produce in vivo has not been fully characterized. The sole class III PI3K, Vps34, produces PI(3)P. Class I PI3Ks and 3-phosphosphorylated phosphatidylinsitols (PIs) have been implicated in cancer: the phosphatase PTEN removes the 3-phosphate from 3-phosphorylated inositides generated by PI3Ks, and PTEN is a tumor suppressor gene mutated in a variety of human cancers (Yamada and Araki, 2001). In addition, mutation, amplication and/or overexpression of some PI3K class I catalytic subunits has been found is some cancers (Katso *et al.*, 2001; Vogelstein and Kinzler, 2004).

Although the class I PI3K family has been implicated in multiple cellular processes (Katso *et al.*, 2001; Vanhaesebroeck *et al.*, 2001), much less is known about the function of the class II PI3K enzymes, PI3KC2α, PI3KC2β, and PI3KC2γ. Both PI3KC2α and PI3KC2β have been implicated in signaling by tyrosine kinase receptors. They are recruited to a signaling complex including the EGF receptor after EGF activation of A-431 cells (Arcaro *et al.*, 2000) and to the stem cell factor (SCF) receptor in small lung cell carcinoma cell lines (Arcaro *et al.*, 2002). The interaction of PI3KC2β with the EGF receptor requires the adaptor protein Grb2, and the SH3 domains of Grb2 bind directly to three proline-rich regions near the amino terminus of PI3KC2β (Wheeler and Domin, 2001). Interestingly, PI3KC2β is required together

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Abbreviations used: EGFR, epidermal growth factor receptor; Erk, extracellular signal-regulated kinase; F-actin, filamentous actin; MEK, mitogen-activated Erk kinase; JNK, c-Jun N-terminal kinase; mTOR, mammalian target of rapamycin; PH, pleckstrin homology; PI, phosphatidylinositol; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; pY, phosphotyrosine; SCF, stem cell factor; SCLC, small cell lung cancer; SH, Src homology.

with class I_A PI3Ks for activation of Akt by SCF (Arcaro *et al.*, 2002).

To investigate signaling by PI3KC2β, we sought to identify its interacting partners. We identified Eps8 as a tyrosine-phosphorylated protein associated with PI3KC2β and established that PI3KC2β is part of a multiprotein signaling complex including Grb2, Eps8, Abi1, and Sos that is assembled and recruited to the EGF receptor, after EGF stimulation. PI3KC2β regulated Rac activity and c-Jun N-terminal kinase (JNK) activation, consistent with the role of the Eps8-Abi1-Sos complex as an exchange factor for Rac. As a result, PI3KC2β expression enhanced membrane ruffling and cell motility. Moreover, PI3KC2β-expressing cells displayed reduced sensitivity to detachment-induced apoptosis and increased proliferation, which involved a Rac-independent signaling pathway.

MATERIALS AND METHODS

Antibodies and Reagents

The following antibodies were used: Akt, Sos1, 9E10 myc epitope, and phospho-Erk (Santa Cruz Biotechnology, Santa Cruz, CA), phosphor-Akt (Ser473), Jun, phospho-Jun, JNK, and phospho-JNK (New England Biolabs, Beverly, MA), Grb2 monoclonal and polyclonal, Shc, Eps8, E-cadherin and pan-Erk antibodies (Transduction Laboratories, Lexington, KY), Rac, and 4G10 anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY), E-cadherin monoclonal antibody (mAb; Zymed Laboratories, South San Francisco, CA).

The rabbit antibody to Abi1 was a kind gift from Paolo Di Fiore (European Institute of Oncology, Milan, Italy). Recombinant human EGF was purchased from R&D Systems (Minneapolis, MN). TRITC-conjugated phalloidin was obtained from Sigma-Aldrich (St. Louis, MO). The Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-*O*-methyl-3-*O*-octadecylcarbonate), the JNK inhibitor I, PD098059, and SB202190 were purchased from Calbiochem (La Jolla, CA).

Cell Lines

A-431 cell lines stably expressing PI3KC2β WT and DN were generated as previously described (Arcaro *et al.*, 2000). The A-431 human epidermoid carcinoma cells were maintained at 37°C, 10% CO_2 under humidified conditions in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, and glutamine. The stock cell cultures were passaged every 4 d. Cells were made quiescent by switching them to DMEM supplemented with 0.1% fetal calf serum (FCS) for 24 h. HEK 293 cells were maintained in DMEM/10% FCS.

Immunoprecipitation and Immunoblotting Analysis

Cells were grown to 80% confluence in 10-cm dishes and starved for 24 h in DMEM supplemented with 0.1% FCS. Where indicated, cells were then stimulated with 1 nM EGF (6 ng/ml) for the indicated time periods. The cells were washed once with ice cold phosphate-buffered saline (PBS) before lysis on ice for 30 min in 1.00 ml of lysis buffer (1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 50 mM Tris.HCl, pH 7.4, 1 mM EDTA, 0.5 mM EGTA) supplemented with protease inhibitors (10 μg/ml aprotinin, leupeptin, pepstatin A, trypsin, and 100 μg/ml phenylmethylsulfonyl fluoride) and phosphatase inhibitors (Sigma phosphatase cocktail I and II solutions). Insoluble material was removed by centrifugation for 30 min (15,000 rpm at 4°C). The Triton-insoluble fraction was analyzed by reextraction of the pellet from above in RIPA lysis buffer (1% Triton X-100, 50 mM Tris.Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 25 mM sodium fluoride, supplemented with 100 mg/ml AEBSF, and 5 mg/ml each of leupeptin, pepstatin A, and aprotinin). The lysates were equalized for protein content before being immunoprecipitated. The total protein concentration was measured using the calorimetric BCA protein assay (Pierce, Rockford, IL). Protein, 800 μg, was incubated with the respective antibodies preadsorbed onto 50 μl of protein A or G agarose beads. After 4-h incubation at 4°C, the immune-complexes were washed three times in ice-cold cell lysis buffer supplemented with 500 mM NaCl and protease inhibitors. The immune-complexes were eluted and denatured by boiling for 5 min in SDS sample buffer (50 mM Tris.HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.25% bromophenol blue). For analysis of total cell lysates, 40 μg of total protein was resolved on 20-cm Hoeffer SDS-PAGE gels (8–12%) and transferred to PVDF membranes in a Tris-glycine-methanol buffer (190 mM glycine, 20% methanol, 25 mM Tris.HCl, pH 7.4) at 150 mA per gel for 7 h at room temperature. After the transfer, blots were processed with the respective antibodies using standard immunoblotting procedures.

Peptide Coupling and Affinity Purification

Peptides were purified by high-pressure liquid chromatography and lyophilized (Alta Bioscience, Birmingham, United Kingdom). The epidermal growth factor receptor (EGFR) peptides and their phosphorylated (p) derivatives corresponding to the EGFR pY992 Shc (DDVVDADEYLIP/DDVVDADEpYLIP) and pY1068 Grb2 (DTFLPVPEYINQ/DTFLPVPEpYING) Src homology 2 (SH2) docking sites were used in the peptide pulldown experiments. The PI3KC2β N-terminal proline-rich peptides, proline-rich peptide (PR) 1 SPPPLPPRAS, PR2 WDTPPLPPRK, and PR3 MPPQVPPRTY were used to map the putative Grb2 SH3-binding site. The lyophilized peptides were resuspended in a sodium phosphate buffer solution (0.1 M sodium phosphate, 150 mM NaCl, pH 7.4, solution). Peptides were immobilized to Actigel-ALD resin (Sterogene Bioseparations, Arcadia, CA) according to manufacturer's instructions. Aliquots of coupled peptides were stored at 4°C in the sodium phosphate buffer supplemented with phosphatase inhibitors and 0.01% sodium azide. For the peptide pulldowns, 1 μg of coupled peptide was incubated with 800 μg of protein at 4°C on a rotating wheel for 1 h. The peptides were washed four times in lysis buffer before subjecting the complexes to SDS-PAGE and immunoblot analysis.

Production of GST-Fusion Proteins

pGEX-2T expression vectors encoding PI3KC2β N-terminal (amino acids 1–300) domain, N-terminal Grb2 SH3 domain, C-terminal Grb2 SH3 domain, Abi1 SH3 domain, Raf Ras-binding domain, and PAK CRIB domain were transformed into *Escherichia coli* BL21 cells. GST fusion protein expression was induced by adding 1 mM IPTG to a 1-l culture of exponentially growing cells at 30°C for 4 h. The bacterial cells were harvested by centrifugation and were either immediately processed or frozen down as bacterial pellets at −70°C. The bacterial pellets were lysed at 4°C in 50 mM Tris.Cl, pH 8.0, 2 mM $MgCl_2$, 10% glycerol, 20% sucrose, 2 mM DTT, 100 μg/ml AEBSF, 1 μg/ml each of aprotinin, leupeptin, and pepstatin A. GST fusion proteins were affinity-purified by incubating the clarified lysate with glutathione Sepharose beads (Pharmacia Biotech, St Albans, United Kingdom) at 4°C on a rotating wheel for 1 h. After extensive washing of the Sepharose beads, bound proteins were eluted by incubating the glutathione Sepharose with 10 mM reduced glutathione in 50 mM Tris.Cl, pH 8.0. The eluted proteins were dialyzed with 100 mM Tris.HCl, pH 7.4, 150 mM NaCl and stored in aliquots in a buffer made up of 20% glycerol, 100 mM Tris.Cl, pH 7.4, and 150 mM NaCl at −70°C.

Rac Activity Assays

A glutathione *S*-transferase (GST)-PAK Cdc42 and Rac-binding region (CRIB) fusion protein was incubated with cell lysates to pull down GTP-bound active Rac. GST-PAK CRIB fusion protein was prepared just before the pulldown assays. (This is all in the section above.) The Sepharose beads with bound GST-PAK CRIB were washed three times in lysis buffer and once in Rac pulldown buffer (50 mM Tris.HCl, pH 7.4, 12 mM $MgCl_2$, 10% glycerol, 100 mM NaCl, 1% NP-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 μg/ml AEBSF). The Sepharose beads were aliquoted (25 μl) into individual tubes for the Rac pulldown assay. Alternatively, GST-PAK CRIB beads were purchased from Upstate Biotechnology. A-431 cells were grown to 80% confluence and made quiescent by culturing them in serum-free medium (0.1%) for 24 h. The cell culture plates were immediately placed on ice, washed once with ice-cold PBS, and lysed for 5 min on ice with 1 ml Rac pulldown buffer (this is given above). The lysates were clarified by centrifugation (14,000 × *g*, 4°C, 5 min), and protein concentration was determined (Bradford assay, Pierce). Equal amounts of lysates were incubated with GST-PAK CRIB fusion protein at 4°C for 30 min. The beads were washed three times in lysis buffer and eluted in Laemmli sample buffer. The amount of Rac pulled down was determined by immunoblotting with anti-Rac mAb (Upstate Biotechnology).

PI3K Assays

For the immune-complex kinase assays, cells were lysed in RIPA buffer for 30 min on ice. The cell lysates were clarified by centrifugation (14,000 × *g*, 4°C, 15 min), and aliquots were taken from the respective tubes for protein quantification. Equal amounts of lysates were incubated with the respective antibodies for 4 h at 4°C. The immune-complexes were washed three times in lysis buffer, once in 50 mM HEPES, pH 7.5, and once in 2× PI3K assay buffer (40 mM Tris.HCl, pH 7.4, 200 mM NaCl). The PI3K assay was initiated by resuspending the immune-complexes in 25 μl of 2× PI3K assay buffer. The phospholipids (PI, PI(4)P, and $PI(4,5)P_2$) were sonicated for 10 min and added at a final concentration of 0.2 mg/ml to the sample tubes. The tubes were incubated on ice for 20 min to allow the substrate to bind to the enzyme. Fifteen microliters of the assay reaction mix (40 mM ATP, 3.5 mM divalent cation, and 10 μCi [γ-^{32}P]ATP) were added to each of the sample tubes on ice before transferring them to room temperature for 20 min. The reactions were terminated by placing the tubes on ice and adding 100 μl of 1 M HCl in addition to 200 μl of a 1:1 solution of chloroform and methanol. The samples were vortexed and centrifuged for 2 min at room temperature. The organic phase was collected and extracted with 80 μl of a 1:1 solution of methanol and 1 M HCl. The tubes were vortexed and centrifuged for 2 min at room

temperature, and the organic phase containing the lipids was transferred to a fresh tube. The lipids were dried down in a speed vacuum before determining the amount of radioactivity incorporated by Cerenkov counting. The lipids were resuspended in 30 μl of a 4:1 solution of chloroform and methanol and spotted onto channeled Silica Gel 60 TLC plates (Whatman, Clifton, NJ) that had been pretreated in 1% oxalic acid, 1 mM EDTA, 40% methanol and baked for 15 min at 110°C. The TLC plates were developed in propan-1-ol, 2 M acetic acid (65:35) for 4 h, and the radiolabeled spots were quantified using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Immunofluorescence and Localization of F-Actin

A-431 cells (1×10^4) were seeded on glass coverslips and grown for 48 h. Coverslips were rinsed in PBS and fixed for 10 min in 4% (wt/vol) paraformaldehyde at room temperature. Cells were permeabilized in a solution of 0.2% Triton X-100 for 5 min and then rinsed with PBS. The coverslips were then incubated with either mouse anti-myc epitope (9E10) or anti-E-cadherin antibodies followed by FITC-labeled goat anti-mouse antibodies and TRITC-conjugated phalloidin for 30 min. Coverslips were mounted in Moviol (Calbiochem, San Diego, CA). Cells were examined by confocal laser scanning microscopy (LSM 510; Zeiss, Welwyn Garden City, United Kingdom). Image files were collected as a matrix of 1024 × 1024 pixels describing the average of eight frames scanned at 0.062 Hz.

Transfections

Control and PI3KC2β-transfected A-431 cells were plated on gelatin-coated 13-mm glass coverslips. Cells were transfected with HA-RacN17 or the appropriate empty vector using Fugene 6 (Roche, Lewes, East Sussex, United Kingdom), according to manufacturer's instruction. After 8 h, cells were serum starved in 0.5% FBS overnight, and treated with EGF (100 ng/ml) for 10 min or were mock-treated. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked in 1% BSA. Immunostaining was performed with the indicated antibodies. FITC-conjugated goat anti-mouse IgGs were used as secondary antibodies. Each experiment was performed at least there time with similar results.

Time-Lapse Video

Control and PI3KC2β-transfected A-431 cells were plated on 100-mm plates in DMEM containing 10% FBS. For live imaging, cells were analyzed with an Olympus IX-70 (Lake Success, NY), collecting images every 30 s for up to 30 min. Images were compiled into movies by using the program Metamorph.

Cell Proliferation

A-431 cell lines (10^5/ml) were grown for 3 d in serum-containing medium (1 or 10%) in the presence or absence of inhibitors. Cell proliferation was analyzed by MTS assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

Induction of Cell Death by Anoikis

Cells grown in attachment were trypsinized and washed once in E4/10% FCS. The cells were then placed in Ultralow attachment plates (Corning; rehydrated according to manufacturer's instruction) at 1×10^6 cells in 3 ml of E4 medium containing 10% FCS and left for 6, 12, or 24 h at 37°C/10% CO_2 in the presence or absence of 50 μM PD098059 or 10 μM SB202190.

Sub-G1 Determination by Flow Cytometry

In E4 medium, 1×10^6 cells containing 10% FCS were centrifuged at 1000 rpm for 5 min and then washed once in PBS before fixation in ice-cold 70% ethanol for 30 min at 4°C. Cells were then washed twice in phosphate-citrate buffer (198 parts of 0.2 M Na_2HPO_4 and 8 parts of 0.1 M citric acid, pH 7.8), and the pellet was resuspended in 50 μl of 100 μg/ml RNAse for 10 min. DNA content was then determined by flow cytometry after addition of 200 μl of 50 μg/ml propidium iodide (Molecular Probes, Eugene, OR).

Annexin V/Propidium Iodide Flow Cytometry Analysis

Cells were resuspended at 1×10^6 cells/ml in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). This suspension, 100 μl, was mixed with 5 μl of fluorescein isothiocyanate-conjugated annexin V reagent (Molecular Probes) and 10 μl of propidium iodide solution (Molecular Probes). The samples were left for 15 min at room temperature in the dark before the addition of a further 400 μl of binding buffer. The samples were analyzed by flow cytometry.

Protein Down-Regulation by RNAi Oligonucleotides

All oligonucleotides were purchased from Dharmacon (Boulder, CO) and used at 70 nM. Cells at 50% confluence were transfected using the Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were then incubated at 37°C/10% CO_2 for 48 h to allow for target down-regulation. The efficiency of protein down-regulation was assessed by SDS-PAGE and Western blotting.

E-Cadherin Neutralization

In a normal or ultralow attachment 96-well plates (Corning Glass, Corning, NY), 10^4 cells per well were treated for 12 or 24 h with 20 μg/ml E-cadherin antibody (clone HECD-1, Zymed, South San Francisco, CA) in E4 medium containing 10% FCS. Cells were either analyzed for sub-G1 by flow cytometry or stained for actin using AlexaFluor 488-Phalloidin (Molecular Probes) according to manufacturer's instructions.

Cell Motility Assays

Cells plated at 25% confluence in 24 well-plates were imaged by phase contrast using a motorized-staged environment controlled Zeiss microscope. Time-lapse images (1 image/10 min/well for 18 h) were acquired using a prior camera linked to the AQM Advance 6 Software (Kinetic Imaging, Liverpool, United Kingdom). Each condition was performed in quadruplicate. Fifteen cells were tracked per replicate using a Kinetic Imaging plug-in and tracks analyzed by Mathematica 5 (Wolfram Research, Champaign, IL) for migration speed and persistence distribution.

RESULTS

PI3KC2β Is Associated with Shc and Grb2

To investigate signaling partners of PI3KC2β, we sought to identify PI3KC2β binding proteins. We have previously shown that PI3KC2β associates with a complex of tyrosine phosphorylated proteins that include EGFR-1 in cells stimulated with EGF (Arcaro *et al.*, 2000). A-431 cells expressing Myc-tagged wild-type PI3KC2β (A-431-C2β WT cells) were stimulated with EGF and lysed, and anti-Myc immunoprecipitates from the Triton-soluble and -insoluble fractions were analyzed for the presence of tyrosine-phosphorylated proteins. In the Triton-soluble fraction, two tyrosine-phosphorylated proteins of about 50 kDa were identified by immunoblotting as the p46/p52 Shc isoforms (Figure 1A). In the immunoprecipitates from the Triton-insoluble fraction, in addition to the p46/p52Shc isoforms, p66Shc was also specifically tyrosine phosphorylated (Figure 1B).

Given that the PI3KC2β N-terminal proline-rich domain mediates the interaction of PI3KC2β with Grb2 (Wheeler and Domin, 2001), we tested whether this domain was also responsible for PI3KC2β interaction with Shc. The N-terminal 300 amino acids of PI3KC2β pulled down both Grb2 and Shc (Figure 1, C and D). The interaction between the p66Shc isoform and the PI3KC2β N-terminal region was constitutive, whereas in the case of the p46/p52Shc isoforms it was induced by EGF (Figure 1C). There was an apparent discrepancy between the constitutive association of the PI3KC2β N-terminal region with p66Shc (Figure 1C) and the EGF-stimulated interaction of PI3KC2β with Shc isoforms (Figure 1, B and G). A possible explanation for these differences may be that the PI3KC2β N-terminal region is not accessible in the full-length enzyme before EGF stimulation, whereas in the pulldown experiments (Figure 1C) the recombinant N-terminal region is free to associate with multiprotein complexes involving p66Shc. Both the isolated N- and C-terminal SH3 domains of Grb2 bound PI3KC2β, but not PI3KC2α (Figure 1E), suggesting a Grb2-PI3KC2β isoform-specific interaction. The PI3KC2β domain that binds to Grb2 was mapped through peptide pulldown assays to two proline-rich stretches encompassing proline region 1 (PR1) (SPPPLPPRAS), and PR3 (MPPQVPPRT; Figure 1F). Interestingly, neither of these proline-rich regions is present in the PI3KC2α and γ enzymes, implying that this association may be unique to the β isoform. Coimmunoprecipitation analysis showed that the association between Grb2 and PI3KC2β was constitutive, whereas the interaction between the p46/p52Shc isoforms and PI3KC2β was EGF-dependent (Figure 1G). These results suggest that PI3KC2β is recruited to the EGFR-1 either indirectly through Grb2 interaction with Shc or directly through Grb2 interaction with the EGFR-1. Consistent with

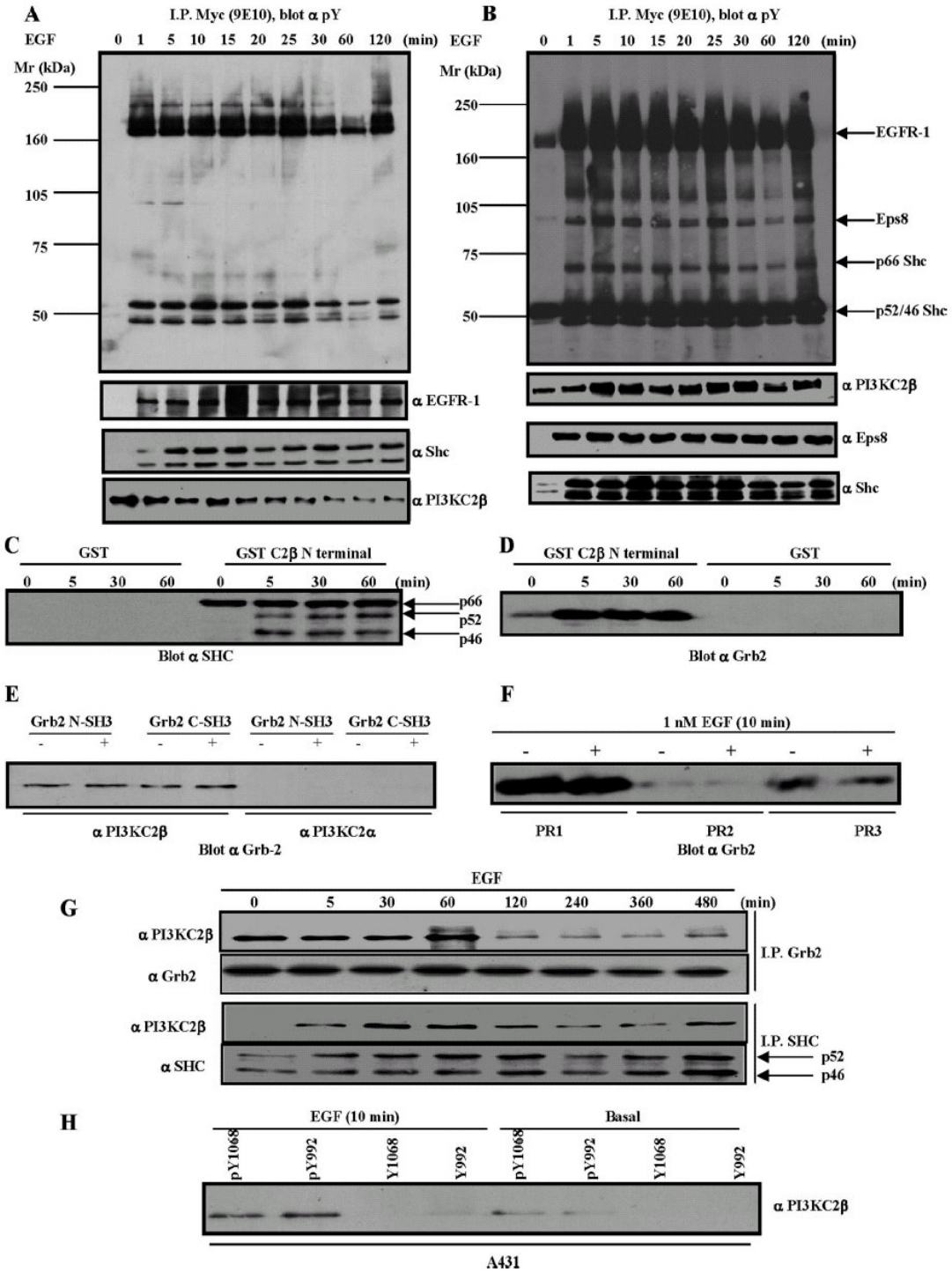

Figure 1. Identification of PI3KC2β-binding proteins. (A) A-431-C2β WT cells were grown to 80% confluence and serum-starved for 24 h before stimulation with EGF for the times indicated. The Triton X-100–soluble (A) and –insoluble fraction (B) were generated as outlined in *Materials and Methods*. PI3KC2β-associated proteins were identified by immunoblotting with the indicated antibodies. (C–E) Lysates from parental A-431 cells were equalized for protein content before immunoprecipitation with the indicated GST-fusion proteins. PI3KC2β N-terminal 300 amino acid domain mediates recruitment of Shc and Grb2 (C and D). Gst-Grb2 pulldowns indicate selective recruitment of PI3KC2β (E). (F) A-431 cell lysates were equalized for protein content before immunoprecipitations with the Actigel coupled PI3KC2β proline-rich peptides, PR1, PR2, and PR3. The amount of Grb2 precipitated by the respective peptides was determined by imuunoblotting.

this hypothesis, EGFR-1 tyrosine-phosphorylated peptides representing residues Y992 and Y1068, the Shc and Grb2 docking sites respectively, immunoprecipitated endogenous PI3KC2β in vitro (Figure 1H).

These observations are in agreement with previous findings that implicated pY992 and pY1068 and Grb2 in the recruitment of PI3KC2β by EGFR-1 (Arcaro *et al.*, 2000; Wheeler and Domin, 2001).

Interaction of PI3KC2β and the Eps8-Abi1-Sos1 Complex

EGF induced a shift in PI3KC2β localization from the Triton-soluble to the -insoluble fraction (Figure 1A, bottom, and B), suggesting an increased association of PI3KC2β with the cytoskeleton and/or signaling microdomains such as rafts. A 98-kDa tyrosine-phosphorylated protein was detected in PI3KC2β immunoprecipitates from the Triton-insoluble fraction and was identified as Eps8 by immunoblotting (Figure 1B). Endogenous Eps8 coprecipitated with PI3KC2β from A-431-C2β WT cells in the insoluble fraction (Figure 1B) and the soluble fraction (Figure 2D). Eps8 has been implicated in the coordination of EGF receptor signaling and trafficking through regulation of Rac and Rab5, respectively, through two mutually exclusive complexes: the Rac GEF ternary complex, Eps8-Abi1-Sos1, and the Rab5-regulatory complex, Eps8-RN-tre (Scita *et al.*, 1999; Lanzetti *et al.*, 2000). PI3KC2β immunoprecipitates prepared from the Triton-insoluble fraction contained Sos-1 and Eps8 (Figure 2A), and Eps8 immunoprecipitates contained PI3KC2β and Abi1 (Figure 2B), indicating that PI3KC2β is complexed with the Eps8 Rac GEF ternary complex (Figure 2B). A-431 cells normally express low but detectable levels of PI3KC2β (Arcaro *et al.*, 2000). Because of the low levels of endogenous PI3KC2β, we were unable to detect the complex in A-431 cells, but we did detect an association of endogenous Eps8 and PI3KC2β in HEK 293 cells, demonstrating that this is a physiological relevant complex (Figure 2C). In contrast, no association between PI3KC2α and Eps8 was observed in HEK 293 cells. Control immunoprecipitations were also performed, which revealed the specificity of the association of Grb2, Shc, Eps8, and Sos-1 with PI3KC2β in A-431-C2β WT cells (Figure 2D).

In the Triton-soluble fraction, PI3KC2β was constitutively associated with Grb2, and EGF stimulation promoted the interaction of the enzyme with the activated EGFR, Shc, Eps8, and Sos-1 (Figures 1A and 2D). In contrast, in the Triton-insoluble fraction, PI3KC2β was constitutively associated with the activated EGFR, Grb2, Eps8, and Sos-1 (Figures 1B, 2, A and B, and 3A). The constitutive association of the complex in the Triton-insoluble fraction was possibly caused by basal activation of the EGFR, either by autocrine TGF-α production, or overexpression of the receptor in A-431 cells (Van de Vijver *et al.*, 1991; Jo *et al.*, 2000).

To identify the domains of PI3KC2β and Eps8 involved in the formation of the complex in A-431 cells, we used pull-down assays with immobilized recombinant GST-domains. The N-terminal region of PI3KC2β interacted with Eps8 in A-431 lysates, independently of EGF stimulation, but no interaction was observed between the PI3KC2β C2 domain and Eps8 (Figure 2E). This data correlated with the observation that this region of PI3KC2β interacts with Shc and Grb2 (Figure 1C). The N-terminal region of Eps8 (residues 1–535) and its SH3 domain both interacted with PI3KC2β in lysates from unstimulated A-431-C2β WT cells (Figure 2F). However, the Eps8 PTB domain failed to bind to PI3KC2β in a comparable manner (Figure 2F). Thus, multiple surfaces of interaction are likely to participate in the formation of an Eps8-PI3KC2β complex.

Assembly of the Grb2-PI3KC2β-Eps8-Abi1-Sos1 Multiprotein Complex

To establish whether the Shc/Grb2/PI3KC2β and the PI3KC2β/Eps8/Abi1/Sos1 complexes were mutually exclusive complexes or one macromolecular complex, we investigated whether Grb2 could coimmunoprecipitate with Eps8. Grb2 immunoprecipitates from the Triton-insoluble fraction of A-431-C2β WT cells contained PI3KC2β and Eps8 as well as Sos1 (Figure 3A). Interaction mapping of the Eps8-Abi1-Sos1 Rac GEF ternary complex has shown that Abi1 acts as a scaffold protein, which holds together Eps8 and Sos1 (Fan and Goff, 2000; Scita *et al.*, 2000). More specifically, the ternary complex is assembled through binding of the Abi1 and Eps8 SH3 domains to the proline rich motifs of Sos1 and Abi1, respectively.

Profile motif scanning of Abi1 and Eps8 with a peptide library–based searching algorithm (Yaffe *et al.*, 2001) indicated a putative proline-rich Grb2 binding site on Abi1 (NIADSPTPPPPPD). GST-Grb2 fusion protein pulldowns demonstrated that Abi1 and Eps8 predominantly associated with the N-terminal SH3 domain of Grb2 (Figure 3B). In addition, endogenous Abi1 coimmunoprecipitated with Grb2 (Figure 3C), consistent with the observed coimmunoprecipitation of Abi1 and Grb2 in an independent study (Fan and Goff, 2000). To determine the physiological relevance of this complex, we investigated whether endogenously expressed Grb2 associated with the Eps8/Abi1/Sos1 Rac GEF ternary complex. Consistent with earlier findings (Fan and Goff, 2000), we observed coimmunoprecipitation of Abi1 with endogenously expressed Grb2, Eps8, and Sos1, as well as PI3KC2β (Figure 3D). Moreover, assembly of the Grb2-PI3KC2β-Eps8-Abi1-Sos1 complex was observed in two independent A-431 clones expressing kinase-dead PI3KC2β (A-431-C2β DN-17 and DN-32) implying that PI3KC2β lipid kinase activity is not required for the assembly of this complex (unpublished data).

These findings suggest that Grb2, Eps8, Abi1, Sos1, and PI3KC2β can assemble into a single complex in A-431 cells upon EGF stimulation (Supplementary Figure 3). We favor a model whereby PI3KC2β is complexed with the Eps8-Abi1-Sos1 ternary complex through direct binding of the N- and C-terminal SH3 domains of Grb2 to Abi1 and PI3KC2β, respectively (Supplementary Figure 3). However, the possibility that additional interactions occur in this multiprotein complex cannot be ruled out. Analysis of PI3K kinase activity in the presence of calcium ions, which are inhibitory to class I PI3Ks but can be utilized by class II enzymes (Arcaro *et al.*, 1998), indicated associated class II PI3K enzymatic activity was in the Grb2, Shc, Eps8, and Abi1 immunecomplexes (Figure 3E). EGF stimulation did not stimulate PI3KC2β activity in Grb2 immunoprecipitates (Figure 3E), which was consistent with the constitutive association between the two proteins (Figures 1G and 2D). In contrast, a marked increase in associated PI3KC2β kinase activity was observed in Shc, Eps8, and Abi1 immunoprecipitates after EGF stimulation, which probably reflects increased or stabi-

Figure 1 (cont). (G) The A-431-C2β WT cell line was induced with 1 nM EGF for the times indicated before immunoprecipitation with Shc and Grb2. Analysis of the respective immune-complexes indicates that Grb2 and the p46/p52Shc isoforms both associate with PI3KC2β. (H) A-431 cell lysates were immunoprecipitated with Actigel coupled phosphorylated and nonphosphorylated peptides representing the EGFR-1 SH2-binding peptides for Grb2 (pY1068/Y1068) and Shc (pY992/992). The amount of endogenous PI3KC2β pulled down was determined by immunoblotting.

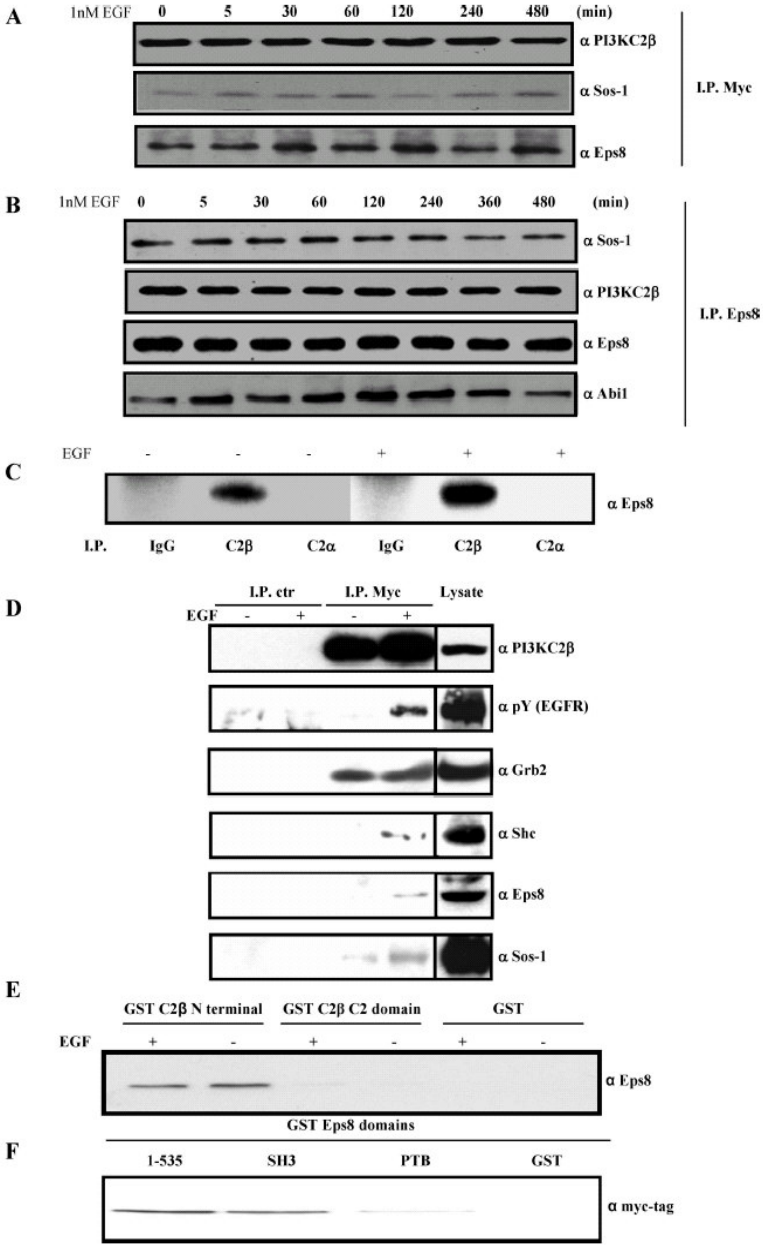

Figure 2. PI3KC2β associates with the Eps8-Abi1-Sos1 Rac GEF ternary complex. (A and B) A-431-C2β WT cells were stimulated with EGF for the indicated time points and then lysed. The Triton X-100–insoluble fractions were solubilized in RIPA buffer and then incubated with antibodies to PI3KC2β (A) or Eps8 (B). Immunoprecipitates were resolved by SDS-PAGE and then Western blots were probed with antibodies to Eps8 or Sos1 (A), or PI3KC2β, Eps8, Abi1, and Sos1. (C) Endogenous PI3KC2β and Eps8 associate in HEK293 cells. Triton X-100–soluble lysates from serum-starved (−) and EGF-induced (+) HEK293 cells were immunoprecipitated with antibodies to PI3KC2α, PI3KC2β, or control rabbit immunoglobulin (IgG). Immunoprecipitates were resolved by SDS-PAGE and Western blotted with antibodies to Eps8. (D) A-431-C2β WT cells were stimulated with EGF for 10 min. The Triton X-100–soluble fraction was immunoprecipitated with immobilized anti-Myc tag or control (mouse IgG) antibodies. Associated proteins were identified by immunoblotting with the indicated antibodies. Cell lysate was analyzed in parallel (20% of total lysate used in the immunoprecipitations). (E) Lysates from quiescent or EGF-stimulated A-431 cells were incubated with immobilized GST, or GST-domains corresponding to PI3KC2β N-terminal region or C2 domain. The samples were analyzed by Western blot with anti-Eps8 antibodies. (F) Lysates from A-431-C2β WT cells were incubated with immobilized GST, or GST-domains corresponding to Eps8 N-terminal region (residues 1–535) SH3, or PTB domain. The samples were analyzed by Western blot with anti-Myc tag antibodies.

lized association of PI3KC2β with the respective proteins (Figures 1 and 2).

PI3KC2β Regulates Rac Activity, Epithelial Adherens Junctions, and Membrane Ruffling

Targeted disruption of Eps8 results in a reduction in platelet-derived growth factor (PDGF)-induced membrane ruffling, Rac activation, and consequently JNK activation in fibroblasts (Scita *et al.*, 1999). Eps8 interacts with Abi1 and Sos to regulate the Rac GEF activity of Sos (Scita *et al.*, 2001; Innocenti *et al.*, 2002, 2003). To establish whether PI3KC2β affected Rac activity, A-431 cells were transfected with either Myc-tagged wild-type PI3KC2β (A-431-C2β WT) or a kinase-dead PI3KC2β that was rendered catalytically inactive by mutation of the highly conserved aspartate (*D*FG) to an alanine residue in the activation domain (D1213A, DN). Lipid immune-complex kinase assays of the respective Myc-tagged PI3KC2β proteins demonstrated that PI3KC2β D1213A (DN) was indeed catalytic inactive (Supplementary Figure 1A). The Cdc42/Rac interactive binding region (CRIB) domain of PAK1 that binds specifically to active GTP-bound Rac (Sander *et al.*, 1998) was used to assess Rac1 activity in the A-431–derived cell lines. In comparison to parental A-431 cells, basal and EGF-stimulated Rac1 activities were significantly

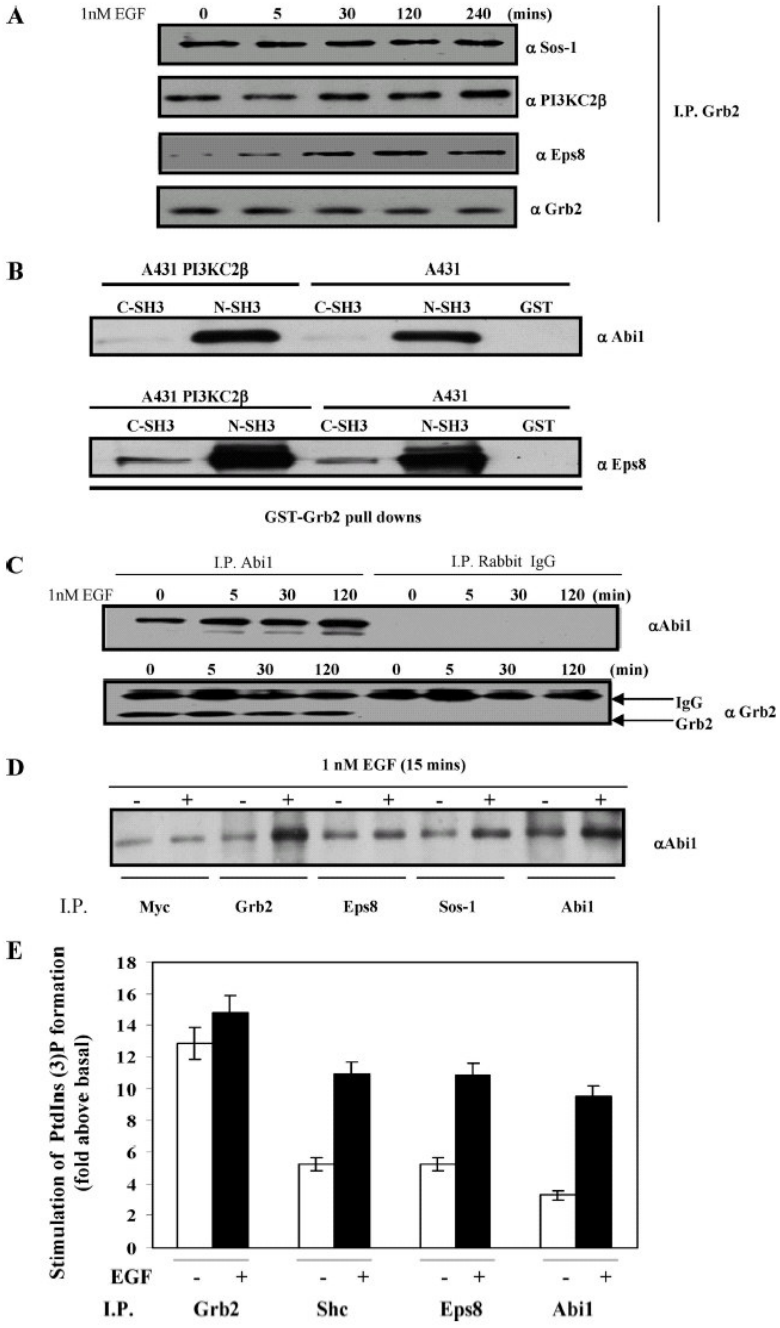

Figure 3. Assembly of the Grb2-PI3KC2β-Eps8-Abi1-Sos multiprotein complex. (A) Grb2 was immunoprecipitated from Triton X-100–insoluble A-431-C2β WT cell lysates and probed with the antibodies to Sos1, PI3KC2β, Eps8, and Grb2. (B) Abi1 interacts with Grb2. Lysates from A-431 and A-431-C2β WT cell line were immunoprecipitated with either the N- or C-terminal SH3 Gst-Grb2 fusion proteins. Precipitated proteins from the respective lysates were immunoblotted with Abi1 and Eps8. (C) Abi1 and control rabbit Immune-globulin complexes from Triton X-100–insoluble lysates of A-431 cells were Western-blotted with anti-Abi1 and anti-Grb2 antibodies. (D) A-431-C2β WT cells were serum-starved for 24 h (−) and stimulated with EGF for 5 min (+). Cell lysates were immunoprecipitated with antibodies to Myc, Grb2, Eps8, Abi1, or Sos1 then Western blotted with antibodies to Abi1. (E) Immune-complex class II PI3K lipid kinase activity was evaluated in the presence of calcium ions as previously described (Arcaro *et al.*, 1998). The results represent the mean of two independent experiments.

increased in A-431-C2β WT cells (Figure 4A). In contrast, Rac1 activity was notably decreased in the A-431-C2β DN cells (Figure 4A) implying that PI3KC2β lipid kinase activity regulates Rac1. Consistent with this observation, EGF-stimulated JNK1/2 activity and c-Jun phosphorylation were upregulated in A-431-C2β WT cells compared with wild-type A-431 cells (Figures 4, B and C). Conversely, JNK1/2 activity and c-Jun phosphorylation were decreased in the A-431-C2β DN cells (Figure 4, B and C). Basal and EGF-stimulated activation of Erk1/2 were attenuated in either A-431-C2β WT or DN cells, as compared with wild-type A-431 cells (Figure 4D). In contrast, activation of Akt1 was comparable between A-431 and A-431-C2β WT cell lines, as assessed by EGF-stimulated induction of Ser473 phosphorylation (Figure 4E). Thus PI3KC2β catalytic activity selectively regulates Rac1 activation in response to EGF in A-431 cells, which impacts on JNK1/2 activity and c-Jun phosphorylation. In contrast, PI3KC2β overexpression attenuates Erk1/2 activation independently of its lipid kinase activity in A-431 cells.

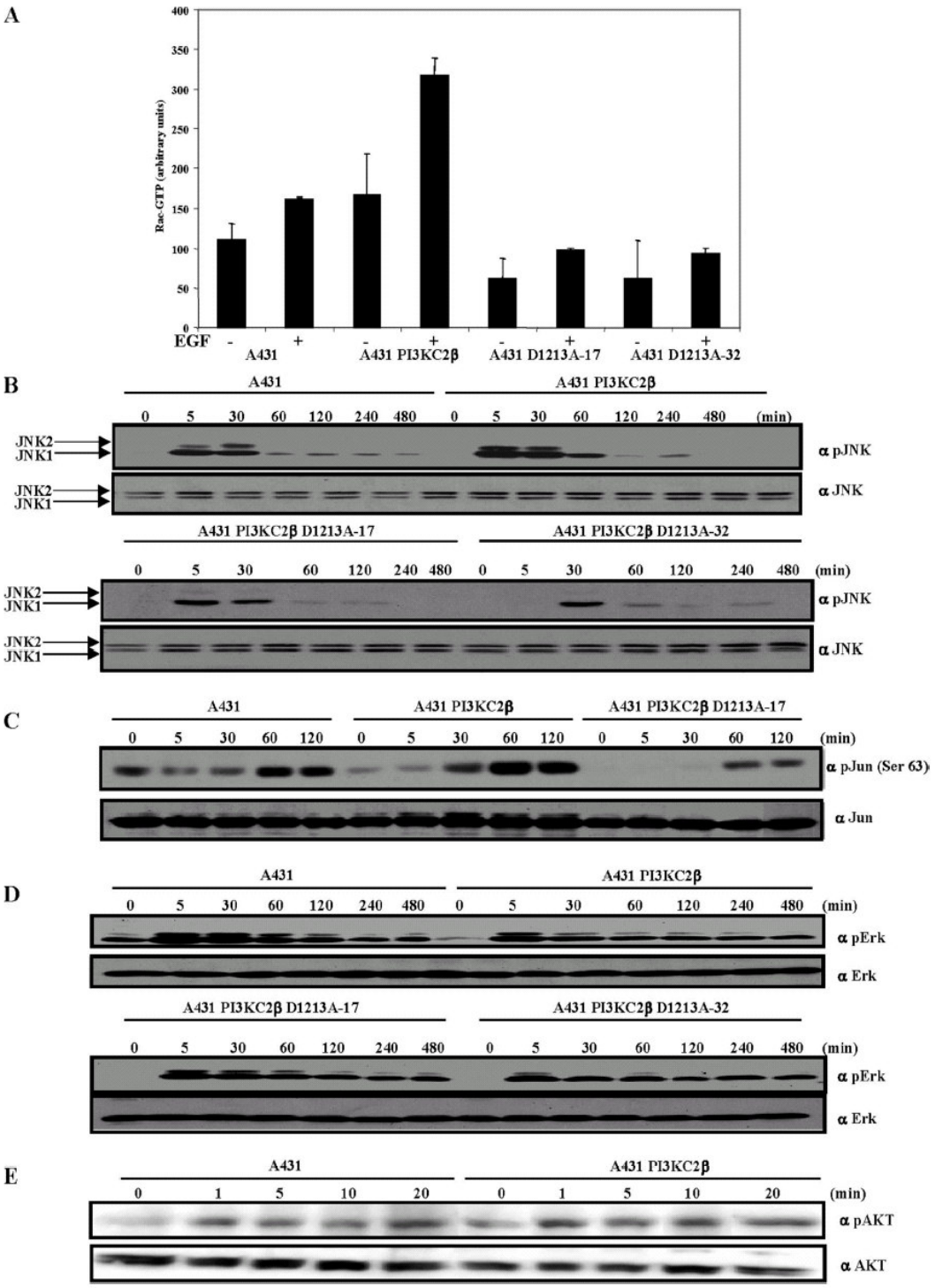

Figure 4. PI3KC2β regulates Rac and JNK activity. (A) PI3KC2β up-regulates Rac activity. Parental A-431 cells, and A-431 expressing wild-type PI3KC2β and kinase-dead PI3KC2β (D1213A-17, D1213A-32) were serum-starved for 24 h and stimulated with 1 nM EGF (+) for 5 min. The cell lysates were equalized for protein content before immunoprecipitation with GST-PAK CRIB. Immunoprecipitated and total Rac was detected by immunoblotting with a Rac mAb. The blots were quantified by densitometry. Data are mean with SD from four experiments. (B–E) A-431, A-431 cells expressing wild-type or kinase-dead PI3KC2β (D1213A-17, D1213A-32) were made quiescent by culturing in serum-free medium for 24 h before stimulation with 1 nM (C–D) or 8 nM (E) EGF for the times indicated. The Triton-soluble

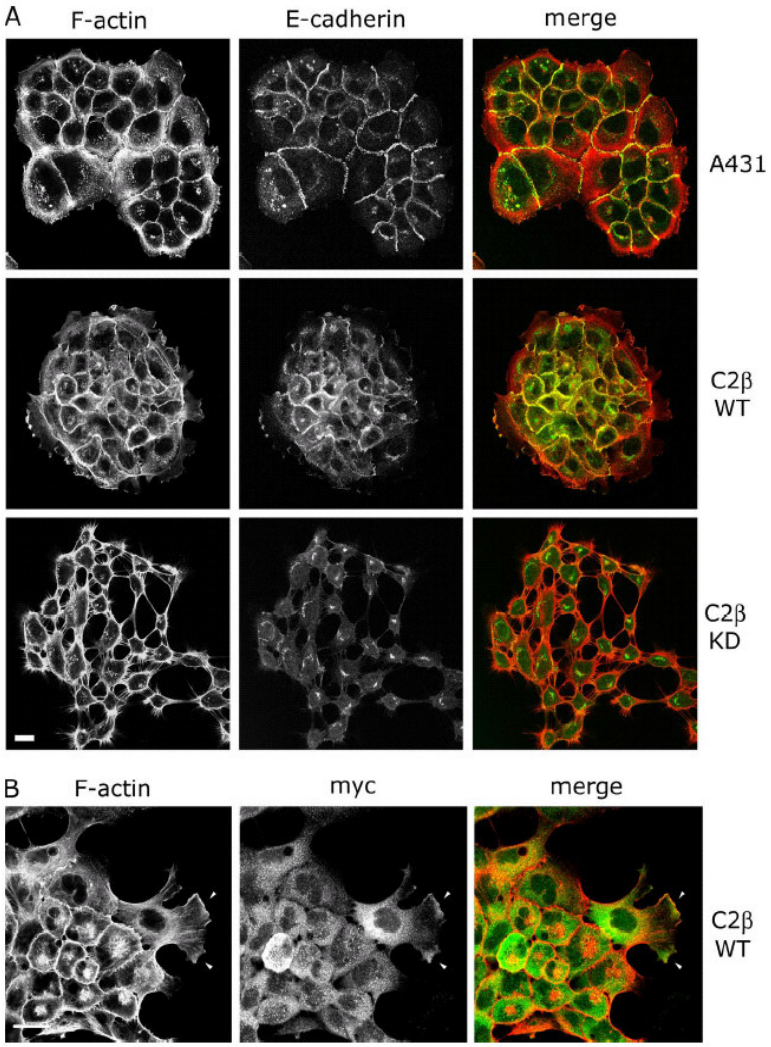

Figure 5. Effects of PI3KC2β on F-actin and adherens junctions. (A) Growing parental A-431 cells, A-431-C2β WT cells (WT), or A-431-C2β DN-32 (KD) cells were fixed and incubated with antibodies to E-cadherin followed by FITC-labeled anti-mouse antibodies and TRITC-labeled phalloidin to localize F-actin. (B) Growing PI3KC2β Myc-6 cells (WT) were fixed and stained with antibodies to the Myc epitope to localize PI3KC2β followed by FITC-labeled anti-mouse antibodies and TRITC-labeled phalloidin to localize F-actin. Arrows indicate PI3KC2β localization in lamellipodia. Bars (A and B), 10 μm.

A striking phenotypic consequence of increased PI3KC2β expression was that the A-431-C2β WT cells formed more compact colonies compared with parental A-431 cells (Supplementary Figure 1B). This phenotypic change is unlikely to be due to clonal variation as it was observed in three independent clones. Conversely, two independent PI3KC2β D1213A-expressing lines (A-431-C2β DN-17 and DN-32) exhibited a reduction in cell–cell contacts (Supplementary Figure 1C and unpublished data).

Analysis of the actin cytoskeleton of A-431-C2β WT cells revealed that PI3KC2β expression induced an increase in F-actin and E-cadherin at cell–cell junctions (Figure 5A), which is in agreement with the observed effects of activated Rac in enhancing adherens junction formation in epithelial cells (Braga *et al.*, 1997; Takaishi *et al.*, 1997). Similar effects were observed in two additional PI3KC2β-expressing cell lines, PI3KC2β Myc-4 and Glu-15 (unpublished data). PI3KC2β showed a predominantly punctate distribution in the cytoplasm and also localized to cell protrusions and ruffling regions of the plasma membrane in cells at the edge of colonies (Figure 5B, arrows), similar to the reported localization of Eps8 and Abi1 (Scita *et al.*, 2001; Stradal *et al.*, 2001). The level of PI3KC2β expression varied within colonies, and in the highest expressing cells there was a clear increase in the level of polymerized actin at cell–cell junctions, in the perinuclear region and in lamellipodia-like extensions. (Figure 5, A and B). The perinuclear accumulation of F-actin may reflect a role for Rac in regulating actin assembly on the Golgi (Fucini *et al.*, 2002). In contrast, A-431-C2β DN cells did not form stable adherens junctions when subconfluent and showed an increase in the level of intracellular perinuclear E-cadherin (Figure 5A). F-actin staining

Figure 4 (cont). extracts were equalized for protein content, resolved on duplicate gels, and probed with the indicated antibodies. The extent of JNK, Jun, Erk, and Akt activation was assessed in the respective cell lines by immunoblotting with phospho-specific antibodies directed against the activated forms of the proteins. The amount of total protein was established by blotting the duplicate blots with antibodies against JNK, c-Jun, Erk, and Akt. The positions of the bands corresponding to JNK1 (p46) and JNK2 (p54) are indicated by arrows.

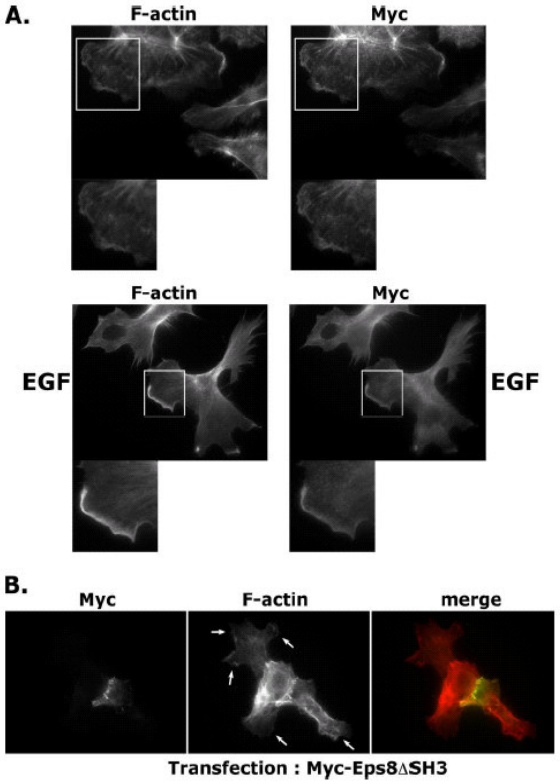

Figure 6. PI3KC2β is localized to lamellipodia in EGF-stimulated A-431 cells. (A) PI3KC2β-expressing cells were incubated for 24 h in a serum-free medium (top) or stimulated with 100 ng/ml EGF for 10 min (bottom). Cells were fixed and stained with phalloidin and anti-Myc antibody to visualize PI3KC2β. (B) A mutant of Eps8 unable to form a complex with Abi1, Eps8ΔSH3, impairs lamellipodia formation in A-431 PI3KC2β-expressing cells. PI3KC2β-expressing cells were transiently transfected with myc-Eps8ΔSH3. After 24 h, cells were fixed and stained with anti-Myc antibody and phalloidin. Lamellipodia, indicated by arrows, were inhibited in roughly 60% of Eps8ΔSH3-expressing cells.

of A-431-C2β DN cells revealed that cells often remained associated via fine F-actin–containing microspikes (Figure 5A). Filopodial extension has been implicated as one of the initial steps in adherens junction assembly (Vasioukhin *et al.*, 2000), where juxtaposed cells initially attach via filopodia to promote actin polymerization and clustering of E-cadherin at the site of cell–cell contact. It is therefore possible that A-431-C2β DN cells retain the ability to extend filopodia, but are unable to activate Rac in order to stabilize cell–cell adhesion.

Because the biochemical analysis had revealed a translocation of PI3KC2β to the Triton-insoluble cytoskeletal fraction upon EGF stimulation (Figure 1B), we sought to further investigate changes in PI3KC2β localization in response to A-431 cell stimulation. Serum-starved A-431-C2β WT cells were stimulated with EGF and localization of F-actin and Myc-tagged PI3KC2β assessed by immunofluorescence. Colocalization of PI3KC2β with F-actin in lamellipodia was observed in EGF-stimulated A-431-C2β WT cells (Figure 6A, bottom panel). An enrichment of PI3KC2β was observed at the leading edge of EGF-stimulated A-431-C2β WT cells, compared with serum-starved cells (Figure 6A, top panel).

Our data had revealed an association of PI3KC2β with the Eps8-Abi1-Sos-1 complex (Figures 1 and 2), implicated in the regulation of Rac activity. To confirm the role of this macromolecular complex in the regulation of Rac-dependent cytoskeletal rearrangements, A-431-C2β WT cells were transfected with an Eps8 mutant carrying a small deletion of four amino acids (538–542) in the SH3 domain (Eps8ΔSH3, Myc tag). This mutant Eps8 was previously reported to be unable to form a complex with Abi1 (Scita *et al.*, 1999). Staining of transfected A-431-C2β WT cells with anti-Myc tag antibodies and phalloidin revealed a strong (60%) decrease of lamellipodia formation in Eps8ΔSH3-expressing cells (Figure 6B). Thus, perturbing the Eps8-Abi1-Sos-1 complex inhibits Rac-dependent cytoskeletal rearrangements in A-431-C2β WT cells.

We next further studied membrane ruffling in A-431 and PI3KC2β transfectants and its dependence on Rac function. Lamellipodia, were detected in at least 70% of EGF-treated A-431 cells, but not (0%) in serum-starved cells (Figure 7A). More than 60% of the PI3KC2β-expressing cells displayed lamellipodia-like extensions in the absence of EGF stimulation. (Figure 7B; see also Supplementary Videos 1 and 2). EGF-stimulated lamellipodia formation was inhibited by transfection of RacN17 into A-431 cells (Figure 7C). Moreover, lamellipodia extension by PI3KC2β-expressing cells in serum-free medium was also inhibited by RacN17 transfection (Figure 7D). Thus PI3KC2β enhances Rac-dependent lamellipodium formation.

PI3KC2β-transfected Cells Display a Rac and PI3K-dependent Increase in Cell Migration

Because of the reported role of Rac in regulating cell motility (Pankov *et al.*, 2005; Yamauchi *et al.*, 2005), we also wanted to investigate the effects of the PI3KC2β-mediated increase in Rac1 activation on A-431 cell migration. To this effect, A-431 and A-431-C2β WT cells were plated at 25% confluence, and their migration pattern was followed by time-lapse microscopy over a period of 18 h. Cell tracks were then analyzed using Mathematica notebooks to determine the Gaussian distribution of migration speeds (Figure 8, A and C) as well as the medium radius from start covered by cells in each condition (Figure 8B). Figure 8, A and C show that PI3KC2β-tansfected cells migrated consistently faster than their parental A-431 counterparts either in the presence or absence of EGF, a growth factor reported to increase A-431 cell motility (Sparatore *et al.*, 2005). We also noticed that the RNAi-mediated down-regulation of Rac1 (Supplementary Figure 2), had opposite effects on A-431-C2β WT and A-431 cell migration. Although the Rac siRNA increased A-431 migration speed, it counteracted the class II PI3K-mediated increase in A-431 cell motility, reverting them to the parental phenotype (Figure 8C). However, these changes in migration speed did not translate into an increase of the area covered by the migrating cells as demonstrated by horizon calculation (Figure 8B). Horizon calculation is a representation of the mean radius of the area covered by the migrating cell from its starting point. Increase in the value of the radius covered represents an increased persistence in cell movement as opposed to randomness. Indeed, only EGF stimulation allowed A-431 and A-431-C2β WT cells to migrate further from their starting point (Figure 8B). This is also consistent with the observation that A-431-C2β WT cells display a fast rate of randomly forming membrane protrusion with respect to A-431 cells (Figure 7, Supplemen-

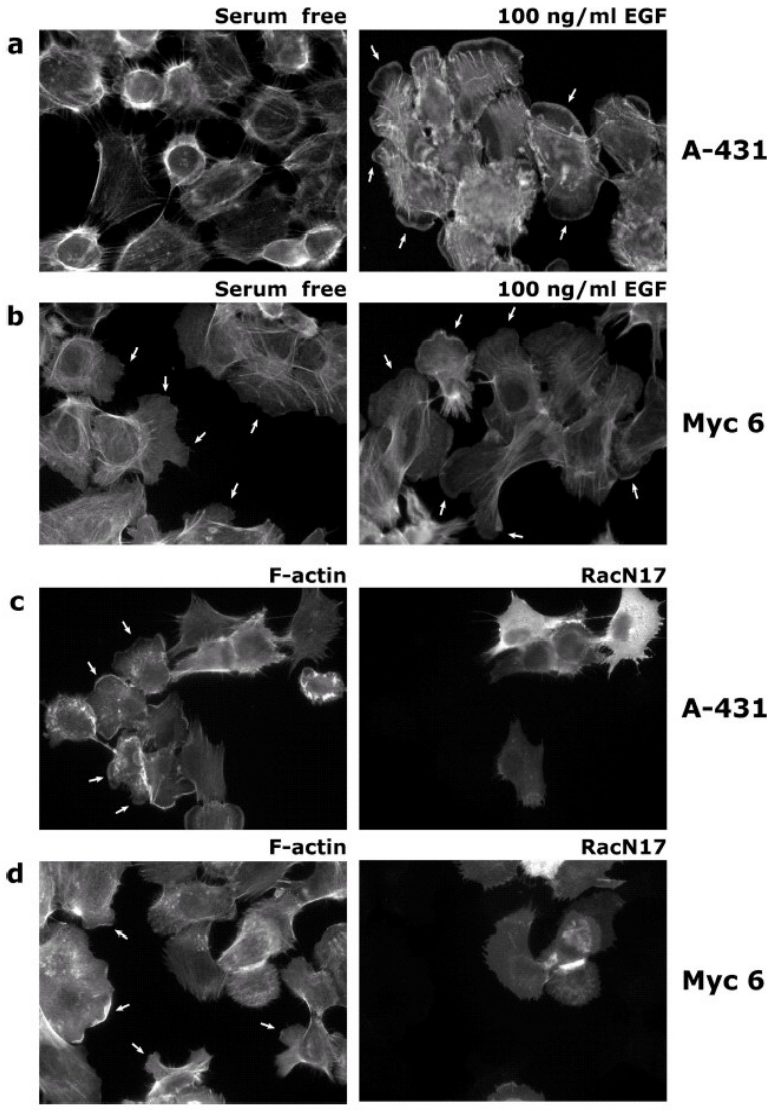

Figure 7. PI3KC2β expression induces lamellipodia constitutively in a Rac-dependent manner. Serum-deprived A-431 control (A) or A-431 PI3KC2β-expressing cells (Myc 6; B) were stimulated with 100 ng/ml EGF for 10 min (right panels) or left untreated (left panels), fixed, and stained with phalloidin to detect F-actin. (C) A-431 control cells were transiently transfected with HA-RacN17, serum-starved, and stimulated with 100 ng/ml EGF. Cells were fixed and stained with anti-HA antibodies and phalloidin. Lamellipodia, indicated by arrows, are inhibited in all RacN17-expressing cells. (D) PI3KC2β-expressing A-431 cells (Myc 6) were transiently transfected with HA-RacN17. Cells were then fixed and stained as in C. Lamellipodia extensions, indicated by arrows, were inhibited in 100% of RacN17-expressing cells.

tary Videos 1 and 2). These data suggest that although PI3KC2β overexpression provides A-431 cells with increased motility speeds, it does not allow for migratory directional persistence. In support of this notion, the migration speed of A-431 cells expressing dominant negative PI3KC2β was significantly decreased, compared with wild-type A-431 cells (Figure 8D). Moreover, the area covered by migrating A-431-C2β DN cells was reduced, compared with control A-431 cells (Figure 8E). Thus, PI3KC2β catalytic activity is also required for migratory directional persistence.

PI3KC2β Expression Increases Cell Proliferation and Renders A-431 Cells Resistant to Anoikis

We next investigated whether the differences in activation of early signaling events observed between A-431 and PI3KC2β-transfected cells (Figure 4) correlated with changes in proliferation and survival of the cells. Proliferation of A-431-C2β WT cells was elevated by ~1.75-fold, as compared with A-431 cells (Figure 9A). This difference was observed both in medium containing low (1%) or high (10%) serum (unpublished data). To investigate whether the increased proliferation of A-431-C2β WT was dependent on the function of JNK or Akt, pharmacological inhibitors of both kinases were tested on both A-431 and A-431-C2β WT cells. The Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-*O*-methyl-3-*O*-octadecylcarbonate) failed to reduce the difference in proliferation between both cell lines at concentrations up to 20 μM (unpublished data). At these concentrations, however, it completely inhibited growth of human neuroblastoma cell lines (unpublished data). A cell-permeable peptide inhibitor of JNK translocation (reported IC_{50} = 1 μM for JNK inhibition) also did not reduce the proliferation of either cell line, although at high concentrations (>25 μM) it reduced proliferation of A-431-C2β WT cells (Figure 9B).

Anoikis is a form of cell death induced by cell detachment from the underlying substratum. Resistance to anoikis is re-

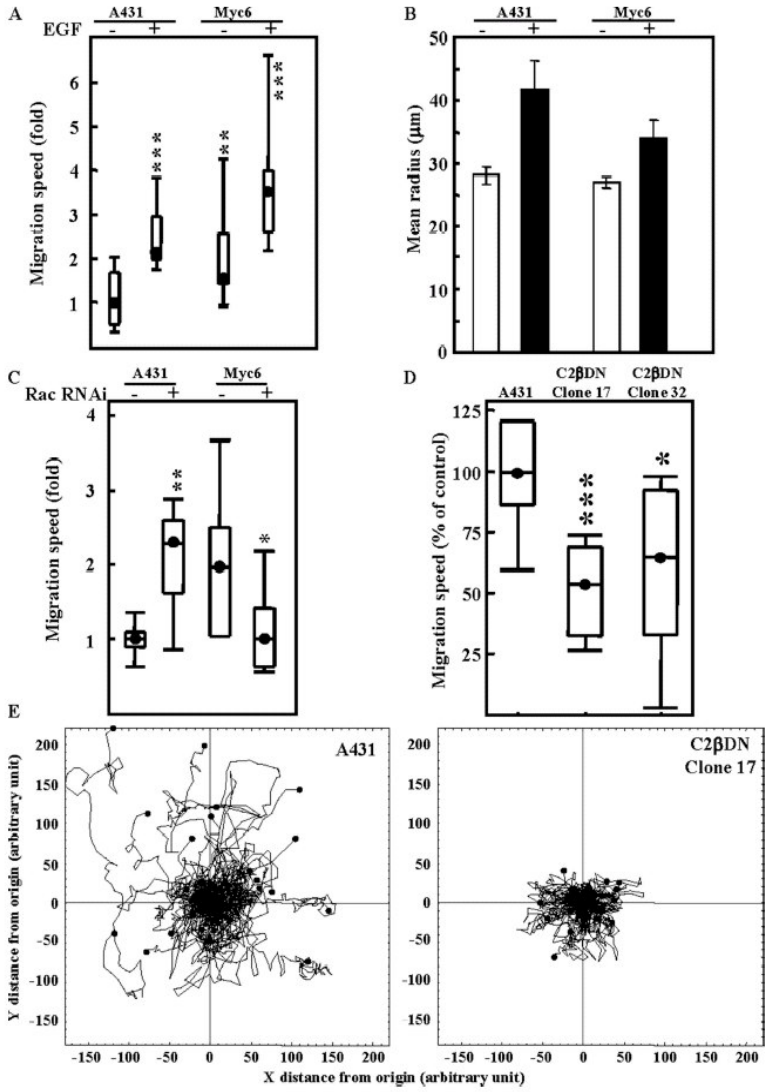

Figure 8. Rac1 RNAi differentially affect A-431 and A-431-C2β WT cell migration. (A and B) A-431 and A-431-C2β WT cells (Myc 6) cells were treated with or without EGF. (C) A-431 and A-431-C2β WT cells were transfected with Rac1 RNAi or scrambled control (−) and left for 48 h in culture to achieve target down-regulation. Cell migration tracks were monitored by live microscopy for 18 h, and migration speed (A and C) or horizon radius (B) was determined using Mathematica. These results are representative of a minimum of three independent experiments. Each condition was performed in quadruplicate using 15 cells per replicate. (D and E) Cell migration tracks of A-431 cells, or A-431 expressing kinase-dead PI3KC2β (C2β DN-17, C2β DN-32) were monitored by live microscopy for 18 h, and migration speed (D), or track paths (E) were determined using Mathematica. Results are representative of a minimum of three independent experiments. Each condition was performed in quadruplicate using 15 cells per replicate. (Significant difference as compared with control A-431 (A and D) or between Rac1 RNAi and scrambled control (C); Student's *t* test: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

quired for tumor cells to establish remote metastasis (Liotta and Kohn, 2004). Therefore, experiments were conducted to investigate whether PI3KC2β expression modified the survival of A-431 cells. A-431 and PI3KC2β-transfected cells were allowed to keep in suspension by plating on ultralow attachment dishes. After 12-h incubation at 37°C, the cells were fixed and stained by propidium iodide for flow cytometry analysis of DNA content. The proportion of dead cells was measured by detecting cells in the sub-G1 fraction of the DNA profile. Figure 9C shows that the proportion of cells in sub-G1 was twice as high in A-431 as in A-431-C2β WT cells (≈30% vs. ≈15%). This difference maintained even at 24-h after detachment (Figure 9D), showing that PI3KC2β expression provides A-431 cells with a long-lasting protection from cell death. In both cell lines, addition of EGF inhibited the induction of anoikis (Figure 9, C and D), as previously described (Jost *et al.*, 2001; Reginato *et al.*, 2003). This decrease in the proportion of cells in sub-G1 correlated with an inhibition of phosphatidylserine exposure in PI3KC2β-transfected cells as demonstrated after Annexin V staining (Figure 9E). Taken together these results demonstrate that expression of class II PI3K desensitizes A-431 cells to the induction of anoikis.

Rac1 and E-Cadherin Do Not Mediate the Resistance of PI3KC2β-transfected Cells to Anoikis

Our previous data had revealed that PI3KC2β-transfected cells display an increase in Rac1 activation and E-cadherin levels (Figures 4 and 5). As these two proteins have been linked to cell survival in other cell systems (Murga *et al.*, 2002; Ferreira *et al.*, 2005), we investigated the possibility that they might participate to Myc6 cells resistance to anoikis. However, down-regulation of Rac1 using oligonucleotide RNAi (Supplementary Figure 2C) had no effect on either A-431-C2β WT or A-431 cell survival after cell detachment (Supplementary Figure 2A). Similarly, E-cadherin inhibition using neutralizing antibodies, although decreasing cell–cell contacts in A-431-C2β WT cells back to the extent

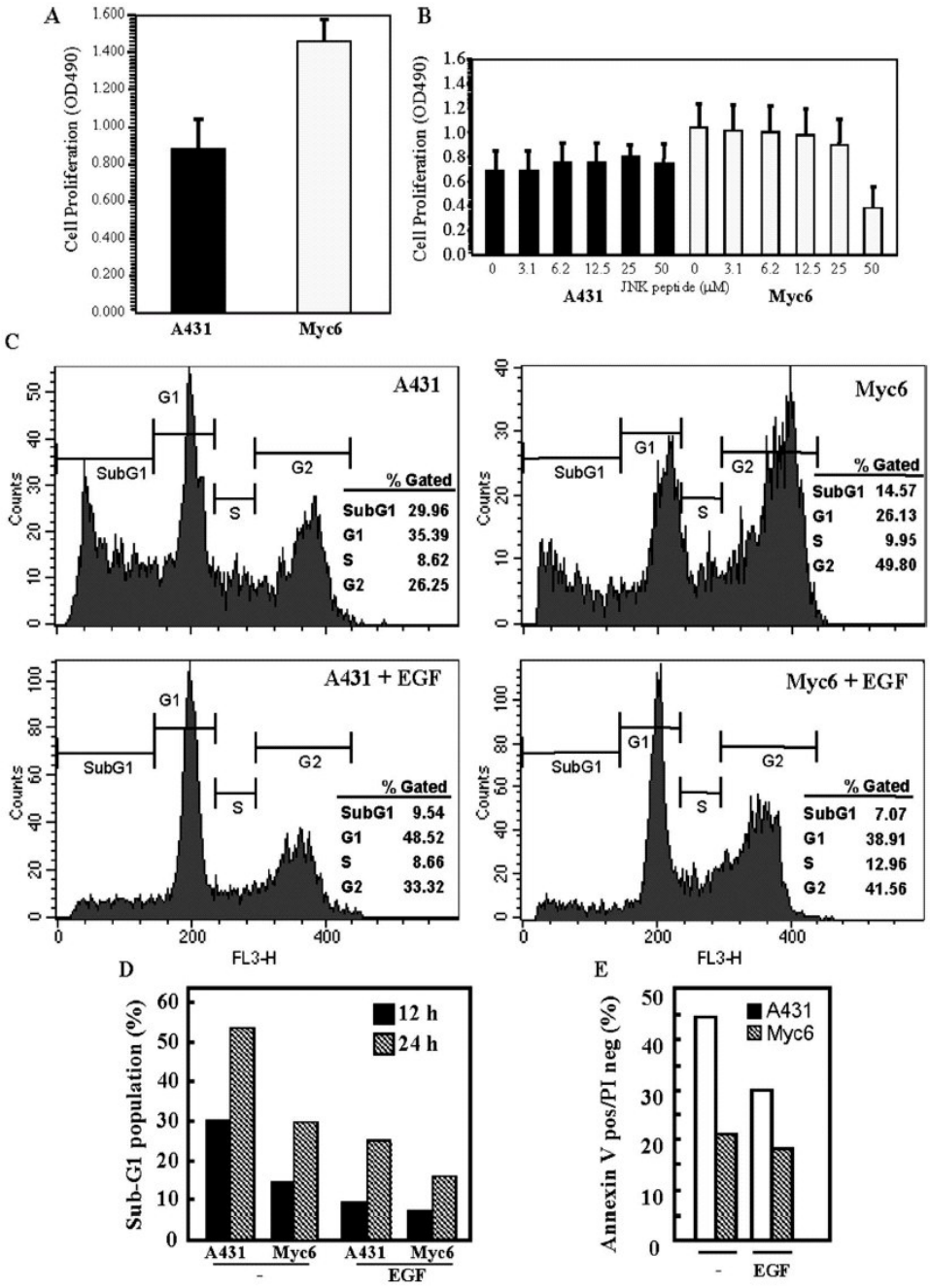

Figure 9. PI3KC2β-transfected A-431 cells display enhanced proliferation and are resistant to anoikis. (A and B) A-431 and A-431-C2β WT (Myc 6) cells were incubated for 72 h in medium containing 1% serum in the absence (A) or presence (B) of increasing concentrations of a cell-permeable JNK inhibitor peptide. Cell proliferation was assessed using a MTS assay. Data are mean with SD from eight repetitions. (C–E) A-431 and A-431-C2β WT (Myc 6) cells were placed for 12 h (C–E) or 24 h (D) in anchorage-free conditions in the presence or absence of EGF. Cells were then stained using propidium iodide alone (C and D) or in conjunction with annexin V (E) and the proportion of cells in SubG1 (C and D) or positive for annexin V only (E) determined by flow cytometry.

observed in A-431 cells (Supplementary Figure 2D), did not impair A-431-C2β WT cells' ability to resist anoikis (Supplementary Figure 2B). Therefore, neither Rac1 nor E-cadherin appear to contribute to the difference in sensitivity to anoikis that exist between A-431 and A-431-C2β WT cells.

Detachment-induced Changes in Early Signaling Molecules Activation and Sensitivity to Anoikis of A-431 and PI3KC2β-transfected Cells

We then hypothesized that differences in the activation of early signaling molecules might be involved in the inhibi-

tion of anoikis after expression of the class-II PI3K. Hence, we determined the phosphorylation status of Erk, Akt, p38, and JNK in A-431 and A-431-C2β WT cells both while attached and 6 h after detachment.

The phosphorylation of p38 appeared unmodified by cell detachment (Supplementary Figure 2E) and incubation of A-431 or A-431-C2β WT cells with the p38 inhibitor, SB202190, although increasing to the same extent the baseline cell death level for both cell lines, did not counteract the effects of class II expression on A-431 survival to anoikis. This suggest that p38 is not involved in the survival advantage of A-431-C2β WT cells.

Although phosphorylation of Erk was equal in attached A-431 and Myc6 cells and remained identical in A-431-C2β WT cells upon detachment, the amount of activated Erk decreased noticeably in A-431 cells after 6 h in anchorage-free incubation (Supplementary Figure 2E). However, inhibition of mitogen-activated Erk kinase (MEK) in A-431-C2β WT cells using PD098059 did not prevent the inhibition of cell death observed in these cells upon detachment (Supplementary Figure 2F). This would suggest that differences in the activation of the MEK/Erk pathway between A-431 and A-431-C2β WT cells are not responsible for their differences in survival.

The phosphorylation of Akt increased in both A-431 and Myc6 cells upon detachment (Supplementary Figure 2E). Although the extent of this increase was similar in the two cell lines, the effects of Akt phosphorylation might still be differentially regulated in A-431 and A-431-C2β WT cells by downstream effectors of Akt. However, Supplementary Figure 2G demonstrates that RNAi-mediated down-regulation of Akt1 and 2 (Supplementary Figure 2C), in isolation or jointly, while increasing the level of cell death in both cell lines, did not prevent the prosurvival advantage of class II PI3K overexpression in A-431-C2β WT cells (Supplementary Figure 2G). Also, the inhibition of the downstream effector of Akt, mTOR (the mammalian target of rapamycin) by rapamycin, did not abolish the survival differential between A-431 and A-431-C2β WT cells (Supplementary Figure 2G). This suggests that changes in Akt phosphorylation cannot account for the class II–mediated inhibition of anoikis.

In contrast to activated Akt levels, the basal phosphorylation of JNK decreased sharply following detachment, both in A-431 and A-431-C2β WT cells (unpublished data). In addition, we found that down-regulation of the JNK upstream kinase, MKK7, by RNAi (Supplementary Figure 2C), while greatly increasing the death toll upon detachment of A-431 cells, had no similar effect on A-431-C2β WT cells survival (Supplementary Figure 2H).

DISCUSSION

The search for potential regulators or effectors of PI3KC2β signaling has identified Abi1, Eps8, and Sos as novel interaction partners of PI3KC2β. Our results show that PI3KC2β forms a complex with Eps8-Abi1-Sos1 upon EGF stimulation and that it regulates Rac activity. Class II PI3Ks preferentially generate PI(3)P and PI(3,4)P_2 in vitro (Arcaro *et al.*, 1998, 2000), but will generate PI(3,4,5)P_3 under certain conditions (Gaidarov *et al.*, 2001), and thus have the potential to generate the same spectrum of 3′-phosphoinositides as class I PI3Ks. We propose that one way through which PI3KC2β can activate Rac by producing phosphoinositides that activate Sos-1 Rac GEF activity in the PI3KC2β-Eps8-Abi1-Sos1 complex. Rac then controls membrane ruffling, cell motility, and cadherin-mediated cell–cell adhesion. Our data suggest that the major determinant in mediating an EGFR-PI3KC2β-Grb2-Eps8 complex is Grb2. Within this macromolecular unit, however, multiple surfaces of interaction are likely to stabilize the entire signaling unit. The PI3KC2β-Grb2-Abi1-Eps8-Sos1 complex could interact with the activated EGFR-1 either directly (pY1068) or indirectly (pY992) through complex formation with Shc. Interestingly, an interaction between the tyrosine phosphorylated p66Shc isoform with PI3KC2β was found specifically in the Triton-insoluble cytoskeletal fraction of EGF-stimulated A-431 cells. This observation may reflect a selective role for PI3KC2β and p66Shc in transducing signals controlling cytoskeletal rearrangements, because a recent report has shown that this Shc isoform plays a role Sos-mediated activation of Rac1 (Khanday *et al.*, 2006). EGFR-1 regulates this macro-molecular complex by stimulating PI3KC2β lipid kinase activity in the complex. Interestingly, a Shc-Grb2-Gab ternary complex has been shown to recruit class I PI3Ks to activated EGFR-1 (Rodrigues *et al.*, 2000), implying that there might be two distinct Shc-Grb2-PI3K class I and II complexes downstream of EGFR-1.

As A-431 cells produce and are constitutively activated by TGF-α acting on the EGFR-1 (Van de Vijver *et al.*, 1991; Jo *et al.*, 2000), it is likely that this induces sufficient PI3KC2β activation in PI3KC2β-overexpressing cells to transduce signals to Rac, without addition of EGF. Indeed, PI3KC2β transfection constitutively stimulated membrane ruffling and cell motility, and both responses were impaired by dominant negative Rac or RNAi. EGF can promote motility and invasion of A-431 cells (Malliri *et al.*, 1998), but is likely that other signals in addition to Rac activation are required for this response, as is the case in HGF-stimulated MDCK cells (Potempa and Ridley, 1998; Sander *et al.*, 1998). Indeed, down-regulation of Rac by RNAi had opposite effects on A-431 and A-431-C2β WT cells, enhancing the migration speed of the parental cells, while inhibiting the response of the transfectants. A possible model is that in A-431 cells Rac1 predominantly controls cell–cell contacts and thus functions as a negative regulator of cell motility. Interestingly, down-regulation of the Rac exchange factor Tiam1 was shown to induce disassembly of cadherin-based adhesions, resulting in enhanced migration of MDCK cells (Malliri *et al.*, 2004). In contrast, in A-431-C2β WT cells, where Rac1 is constitutively activated, it may function predominantly as a regulator of cell motility and membrane ruffling.

PI3K has been implicated in the regulation of E-cadherin–mediated adherens junction assembly in a number of independent studies (Nakagawa *et al.*, 2001; Kovacs *et al.*, 2002). However, these studies have used the generic PI3K inhibitors, wortmannin and LY294002, which do not discriminate between class I and class II PI3K-regulated events. Gene targeting studies have recently revealed a specific role for class I_A PI3K isoforms in cytoskeletal rearrangements, such as membrane ruffling stimulated by PDGF (Brachmann *et al.*, 2005). The relative contribution of class II or class I PI3Ks to Rac activation is thus likely to depend on the receptor utilized as well as the cellular context. Previous work has shown that the EGF receptor couples to class I_A PI3K signaling only in a cell type–specific manner (Arcaro *et al.*, 2000). Sos-1 can contribute to Rac activation both directly, through its Rac GEF domain and indirectly through activation of Ras by its Ras GEF domain and subsequent PI3K activation (Rodriguez-Viciana *et al.*, 1997). We have previously shown that in contrast to class I PI3Ks, PI3KC2β is not activated by Ras (Arcaro *et al.*, 1998). Therefore, although class I PI3Ks mediate Ras-dependent activation of Rac, PI3KC2β is likely to couple growth factor receptor signals to the Rac machinery in a Ras-independent manner. Although more work will be required to define the exact interplay of

the respective components, it is feasible that the divergent influence of Ras regulation of classes I and II PI3Ks upstream of Rac will also influence the cellular outcome. In conclusion, our results identify novel effectors of PI3KC2β cellular function and demonstrate for the first time a role for a class II PI3K in cytoskeletal activation by defining a molecular link between the EGFR-1, PI3KC2β, and Rac. A recent report has shown that PI3KC2β controls cell migration in response to lysophosphatidic acid (LPA) in human cancer cells such as HeLa cells (Maffucci *et al.*, 2005). It is at present unclear whether the same molecular mechanisms are involved in the ability of PI3KC2β to transduce signals from serpentine (LPA) or receptor tyrosine kinases (EGFR), although LPA is a known activator of Rac (Moolenaar *et al.*, 2004).

In contrast to its role in the regulation of cell motility and membrane ruffling, Rac1 appeared to play no role in PI3KC2β-induced protection of A431 from anoikis. The anti-apoptotic effect of PI3KC2β overexpression was also independent of Akt signaling, mTOR, and E-cadherin–mediated cell–cell contact. The observation that PI3KC2β-transfected cells display enhanced growth may thus be in part a result of this difference in survival. In agreement with the anoikis data, pharmacological inhibitors of JNK or Akt failed to affect the enhanced growth response observed in PI3KC2β-overexpressing A-431 cells. Pharmacological inhibition of MEK also failed to alter the response, but PI3KC2β-transfected A-431 cells displayed a more sustained activation of Erk1/2 than A-431 cells upon detachment. Thus, one cannot exclude the possibility that the differences in Erk activity between the two cell lines occur via a MEK-independent pathway (for example, protein kinase C [PKC] isoforms, phophatases; Grammer and Blenis, 1997; Bapat *et al.*, 2001; Kinkl *et al.*, 2001; Tajima *et al.*, 2005). EGF-stimulated activation of JNK was enhanced in PI3KC2β-transfected cells, correlating with the increase in Rac1 activation in these cells. Moreover, our data show that down-regulation of the JNK upstream kinase, MKK7 by RNAi, while greatly increasing detachment-induced apoptosis of A-431 cells, had no similar effect on A-431-C2β WT cells' survival. In line with the reported role of the JNK pathway in the survival of some cell systems (Wang *et al.*, 1999; Gururajan *et al.*, 2005), these data suggest that inhibition of JNK activity is toxic to A-431 cells and that class II overexpression might prevent this toxicity in A-431-C2β WT cells.

Although the present study was restricted to the A-431 cell line, we have found evidence that PI3KC2β interacts with Eps8 and regulates Rac activity in other human cancer cell lines. In human small cell lung cancer (SCLC) cell lines, which overexpress PI3KC2β, compared with normal lung epithelial cells (Arcaro *et al.*, 2002), an interaction between Eps8 and the class II PI3K was observed. SCLC cell lines expressing high levels of endogenous PI3KC2β had increased Rac activity and displayed enhanced JNK activation, compared with cell lines with low levels of PI3KC2β expression. Moreover, expression of kinase dead PI3KC2β in SCLC cells inhibited Rac and JNK activation (unpublished observations). Thus, the interaction of PI3KC2β with Eps8 involved in Rac activation may be of significance in various human cancer cells.

Together, the present report provides novel insights into the functions of class II PI3Ks in the context of human cancer. Human tumor cells have been reported to overexpress class II PI3K isoforms, such as PI3KC2β (Arcaro *et al.*, 2002). The ability of PI3KC2β to enhance migration and protect cells from anoikis may provide a crucial contribution to the malignant phenotype of various human cancers.

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3.2.3 Targeting PI3KC2□ impairs proliferation and survival in acute leukemia, brain tumours and neuroendocrine tumours

(submitted to British journal of cancer)

Running Title: PI3KC2β in human cancers

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ABSTRACT

Eight mammalian phosphoinositide 3-kinase (PI3K) isoforms exist which are subdivided into three classes. While much attention has been given to the class I isoforms, little is known about the functions of class II PI3Ks in human cancer. The expression pattern and functions of the PI3KC2β isoform were investigated in a panel of tumour samples and cell lines. Over-expression of PI3KC2β was found in subsets of tumours and cell lines from acute myeloid leukemia (AML), glioblastoma multiforme (GBM), medulloblastoma (MB), neuroblastoma (NB), and small cell lung cancer (SCLC). Specific pharmacological inhibitors of PI3KC2β or small interfering RNA (siRNA) impaired proliferation of a panel of cell lines and primary cultures from AML, brain tumours and neuroendocrine tumours. Inhibition of PI3KC2β also induced apoptosis in AML and GBM cell lines and sensitised the cells to chemotherapeutic agents. Furthermore, PI3KC2β inhibition impaired the phosphorylation of downstream signalling mediators in AML. Together, these data show that PI3KC2β contributes to proliferation and survival in AML, brain tumours and neuroendocrine tumours and may represent a novel target in these malignancies.

Keywords: PI3KC2β, pharmacological inhibition, cell proliferation, migration

INTRODUCTION

Phosphoinositide 3-kinases (PI3Ks) play an essential role in the signal transduction events initiated by the binding of extracellular signals to their cell surface receptors (Katso et al., 2001; Vanhaesebroeck et al., 2001). The cellular responses controlled by PI3Ks are extremely diverse, including mitogenesis and proliferation, protection from apoptosis and cell motility (Katso et al., 2001; Vanhaesebroeck et al., 2001). There are eight known PI3Ks in humans, which have been subdivided into three classes, based on structural homology and *in vitro* substrate specificity (Vanhaesebroeck et al., 1997a; Vanhaesebroeck & Waterfield, 1999). Class I_A comprises three highly homologous isoforms, p110α (Hiles et al., 1992), p110β (Hu et al., 1993) and p110δ (Chantry et al., 1997; Vanhaesebroeck et al., 1997b), which exist as a heterodimeric complex with a regulatory subunit containing two Src homology-2 (SH2) domains, mediating enzyme association with phosphotyrosine residues in the cytoplasmic domains of activated polypeptide growth factor receptors (Inukai et al., 1996; Otsu et al., 1991; Pons et al., 1995). All class I PI3Ks function as PtdIns(4,5)P_2 3-kinase *in vivo*, upon activation by receptor tyrosine kinases or serpentine receptors (Hawkins et al., 1992; Stephens et al., 1991). PtdIns(3,4,5)P_3 serves as a docking site for the serine/threonine protein kinase phosphoinositide-dependent protein kinase-1 (PDK-1) which is activated upon binding (Alessi et al., 1997). Several protein kinases have been identified as downstream targets of PDK-1, such as the serine/threonine protein kinase B (PKB) /Akt, which is a key regulator of cell survival, ribosomal protein S6 kinase (S6K), which stimulates protein synthesis and cell growth, glycogen synthase kinase-3 (GSK-3), a key regulator of glycogen synthesis, and a subset of protein kinase C (PKC) isoforms (Le Good et al., 1998; Vanhaesebroeck & Alessi, 2000).

Class II PI3Ks comprise the Drosophila PI3K_68D/Cpk (MacDougall et al., 1995; Molz et al., 1996), mouse Cpk-m (Molz et al., 1996), and human PI3KC2α (Domin et al., 1997), PI3KC2β (Arcaro et al., 1998) and PI3KC2γ (Misawa et al., 1998; Ono et al., 1998). The hallmarks of class

II family members are a substrate specificity restricted to PtdIns and PtdIns(4)P *in vitro*, and a conserved C-terminal C2 domain, involved in phospholipid binding. Recent studies have started to investigate the regulation and functions of class II PI3Ks *in vivo*. Indeed, several reports have shown that class II PI3Ks are downstream targets of activated receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), c-Kit and insulin receptor (IR) (Arcaro et al., 2002; Arcaro et al., 2000; Brown et al., 1999; Katso et al., 2006). Studies in *Drosophila melanogaster* have also revealed a role for the class II PI3K_68D in cell differentiation downstream of the EGFR (MacDougall et al., 2004). Furthermore, recent reports have documented a role for PI3KC2β in cell migration in mammalian cells via activation of Rho family GTPases and in human cancer cells (Domin et al., 2005; Katso et al., 2006; Maffucci et al., 2005). Studies by others have described a role for PI3KC2α in Rho activation and contraction in vascular smooth muscle cells (Wang et al., 2006). Analysis of a conditional knock-out mouse of *PIK3C2B*, however, revealed no obvious phenotype despite a fairly ubiquitous deletion of *PIK3C2B* (Harada et al., 2005).

The importance of PI3K signalling in cancer is highlighted by the fact that mutations in the tumour suppressor gene phosphatase and tensin homologue (*PTEN*) occur frequently in human tumors. PTEN is a phosphatase that antagonises the action of PI3K by de-phosphorylating the D-3 position of polyphosphoinositides (Cantley & Neel, 1999; Sansal & Sellers, 2004). Moreover, recent reports have described activating mutations in the *PIK3CA* gene encoding the catalytic p110α isoform of PI3K in a variety of human cancers including breast, colon and ovarian cancers (Broderick et al., 2004; Samuels et al., 2004; Weir et al., 2004).

In the present report we have evaluated the expression of the class II PI3KC2β isoform in a panel of primary human tumours and cell lines. Furthermore, we have used isoform-specific pharmacological inhibitors and RNAi to inhibit this enzyme in human cancer cell lines. We show for the first time that PI3KC2β is over-expressed in acute myeloid leukemia, brain

tumours and neuroendocrine tumours and that inhibiting this class II PI3K decreases proliferation and survival in cell lines from these cancers.

MATERIALS AND METHODS

Reagents and antibodies

Antibodies and reagents were purchased from the following companies: The PI3KC2β antibody was described in (Arcaro et al., 1998). Caspase-3, PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA); activated Akt/PKB (Ser473), activated JNK (Thr183/Tyr185), activated S6 protein (Ser235/236) (Cell Signalling Technology, Danvers, MA, USA); β-actin, β-tubulin (Sigma-Aldrich, St Louis, MO, USA); siGENOME™ siRNA (Dharmacon, Lafayette, CO, USA);. (PIramed, Berkshire, UK); Etoposide (Calbiochem, La Jolla, CA, USA); Doxorubicin (Pfizer AG, Zurich, CH). PI701 (YM185453) and PI702 (YM182832) were provided by Piramed Pharma Limited and their synthetic details will be the subject of a separate publication.

Apoptosis

For detection of apoptosis, cells were incubated for 16-24 hrs in the presence or absence of inhibitors. The cells were lysed and caspase-3 activity was measured using the CaspACE Assay System (Promega). Additionally, samples were analysed by SDS-PAGE and Western blot with anti-caspase-3 or anti-poly(ADP-ribose) polymerase (PARP) antibodies.

Cell culture

Acute myeloid leukemia, neuroblastoma, glioblastoma, medulloblastoma, small cell lung cancer and mammary epithelial cell lines were grown in RPMI or DMEM (Life Technologies/InvitrogenCarlsbad, CA, USA) with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin/L-glutamine, and passaged every 3-5 days. DAOY, D341, D425 and D458 medulloblastoma cell lines were grown in MEM Zinc option (Richter's modification) medium supplemented with 10% FCS. For growth factor stimulations cells were incubated overnight in their growth medium with low serum (0.5-1% v/v) or Optimem medium (Life Technologies/Invitrogen) and washed with serum-free medium prior to incubation with growth

factors. Heparinized peripheral blood or bone marrow samples were obtained from adult patients with AML. Blast cells were isolated as described previously (Doepfner et al., 2007). Type II human lung pneumocytes (Pardo et al., 2001) were maintained in DCCM-1 medium supplemented with 10% new born calf serum.

Cell proliferation

Cell lines (5 x 10^3 cells/well) were seeded in 96-well plates and grown for 72h in serum (10%)-containing medium in the presence or absence of inhibitors. The number of viable cells was analysed by means of an MTS assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Data are mean with SD from 8 repetitions.

Dissociation of brain tumours

Human brain tumours were removed from 4 patients who underwent surgery for tumour resection at the University Hospital Zurich. The procedures were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Canton Zurich. Following removal, tumour tissue was immediately placed in a petri dish, minced mechanically and digested enzymatically with collagenase D and DNase I (Roche Applied Science, Rotkreuz, Switzerland) for 1 hour at 37°C while being stirred with a magnetic bar. The dissociated cells were then sequentially filtered through 100 and 70µm cell strainers (BD Falcon, BD Biosciences, Basel, Switzerland) to remove any tissue debris. Erythrocytes were removed by resuspending and incubating the cells in ice-cold ACK buffer (17 mM Tris–HCl [pH 7.2] containing 144 mM NH4Cl) for 10 minutes on ice. The cells were washed in PBS and plated in DMEM (Life Technologies/Invitrogen) supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS) and gentamycin (20mg/ml) and passaged every 3-5 days by trypsinization.

DNA Microarray

Total RNA was extracted from 60 medulloblastoma samples (MB) and 3 cell lines (D283, D341, and DAOY) using the Trizol reagent (Invitrogen). After DNAse treatment and RNA purification (RNeasy Micro kit, Qiagen), gene expression profiles were obtained on the Affymetrix HG-U133 Plus 2.0 array that contains more than 54000 probe sets for transcripts and variants. Expression data for 9 normal cerebellums analyzed on the same Affymetrix array version were obtained from Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Gene expression data for the samples were normalized using the GCRMA procedure. PI3KC2β expression levels for the samples are presented (unlogged data).

Isolation of RNA from tumour samples and RT-PCR

For primary neuroblastoma samples ethical approval to use residual tissue was obtained. RNAlater-preserved tumour tissue was available in the Swiss Pediatric Oncology Group tumour bank. All diagnoses were confirmed by histological assessment of the tumour specimen obtained at surgery. Neuroblastoma tissue was disrupted with a sterile disposable tissue grinder (Sage Products, Cary, IL, USA) and homogenised in guanidinium isothiocyanate-containing buffer. Total RNA of cell lines or tumour tissue was isolated using the RNeasy kit (Qiagen, Santa Cruz, CA, USA) according to the manufacturer's protocol. Total RNA (3 µg) from each tumour sample was converted into cDNA using the SuperScript™ First-Strand Synthesis System for PCR according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). mRNA expression of PI3KC2β and 18S (internal control gene) was measured in tumour samples and cell lines by TaqMan® Assay-on-Demand™ Gene Expression products (Applied Biosystems, Foster City, CA, USA). The following primers were used (gene - assay ID): PI3KC2beta - Hm00153248_m1; eukaryotic 18S rRNA - Hs99999901_s1. Three replicates were run for each sample in a 96-well format plate. Gene expression assays consisted of a

FAM™ dye-labelled TaqMan® MGB probe and two PCR primers. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 10 min and a 50-cycle countdown at 95°C for 15 s and 60°C for 1 min. Each sample was normalised on the basis of its 18S rRNA content. Relative mRNA expression levels were calculated using the comparative threshold cycle (CT) method (Giulietti et al 2001).

PI3K Assays

Recombinant human PI3KC2β was expressed as a glutathione *S*-transferase (GST)-fusion protein in SF9 insect cells and purified as described previously (Arcaro et al., 1998). Recombinant class I PI3K isoforms were expressed and purified likewise. PI3K activity of the different PI3K isoforms was assayed essentially as described (Hayakawa et al., 2007).

SDS-PAGE and Western blot analysis

Cellular lysates were prepared as previously described (Guerreiro et al., 2006) separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences), and immunoblotted with various antibodies according to the manufacturer's protocol. Chemiluminescence was used for visualization using the enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Biosciences) according to the manufacturer's protocol.

Transient expression in AML cells

AML cells were transfected with small interfering RNA (siRNA) targeting PI3KC2β using the Amaxa Nucleofector system (Amaxa biosystems, Gaithersburg, MD, USA) according to the manufacturer's protocol. Cell Line Nucleofector Kit V was used and program V-001 applied. After 48h cells were lysed in cell lysis buffer in order to visualize protein expression by SDS-PAGE and Western blotting. Besides, cells were

analysed for cell proliferation and apoptosis by MTS assay and Caspase-3 measurement 72h after transfection.

Wound healing assay

EpH4 murine epithelial cells stably transfected with the Ha-*Ras* oncogene and induced by TGF-β1 to undergo epitheliomesenchymal transition (EMT) *in vivo* to establish FibRas cells were previously described in (Maschler et al., 2005). For wound healing assays, cells were plated in 12-well culture plates in complete medium and grown to confluency. A wound was created by scraping cells with a 200µl tip. The migration rate was monitored for eight hours by phase contrast microscopy (Leica DM IRBE Inverse, Widefield) either in the presence or absence of inhibitors.

RESULTS

Expression of PI3KC2β in tumour samples and cell lines

Previous reports had documented increased expression of PI3KC2β in leukemia, glioblastoma and lung cancer cell lines and tumours (Arcaro et al., 2002; Knobbe & Reifenberger, 2003; Qian et al., 2002). Therefore, the expression of PI3KC2β was investigated in a panel of tumours and cell lines from AML, neuroendocrine tumours (SCLC and NB) and brain tumours (MB and GBM). In AML, PI3KC2β was highly expressed in a subset of AML blasts and cell lines (Figure 1A, left panel). Interestingly, PI3KC2β expression was much lower in non-leukemic bone marrow cells and immortalised B cells (Figure 1A) indicating that AML blasts and cell lines over-express the enzyme. We also reanalysed cDNA microarray data from a previously published study in AML (Valk et al., 2004). PI3KC2β mRNA expression was found to be higher in certain groups of AML, depending on molecular and cytogenetic abnormalities. AML categories displaying increased expression included FLT3-ITD and EVI1 (77.37; 99.21), as compared to FLT3-TKD (41.51). Increased expression of PI3KC2β was also found in AML with the cytogenetic abnormalities -7 (99.72), when compared to +8, 11q13, t(8;21), idt(16), or NN (46.86; 39.35; 54.40; 19.80; 60.03). In contrast, there were no significant differences in expression of PI3KC2β mRNA between AML French-American-British (FAB) classes.

Protein expression analysis of SCLC cell lines revealed elevated PI3KC2β expression in 2/4 cases when compared to a normal Type II pneumocyte cell line (PN) (Figure 1B, left panel). TaqMan analysis of *PIK3C2B* expression confirmed that mRNA levels are predictive of protein expression (Figure 1B, right panel).

In neuroblastoma (NB) cell lines, Western blot analysis revealed broad expression of PI3KC2β (Figure 2A). Upon TaqMan analysis *PIK3C2B* was found to be over-expressed at the mRNA level when compared to normal human adrenal gland (5/8 samples with >2-fold expression) (Figure 2B). In primary tumours from children under the age of one year PI3KC2β showed

increased expression (Figure 2C). This distribution was mirrored in data obtained from TaqMan analysis, where increased mRNA expression was found in the same patient subgroup (3/19 samples with >2-fold expression) (Figure 2D).

In glioblastoma multiforme (GBM) cell lines and *ex vivo* cultures PI3KC2β was found to be over-expressed in a subset of samples when compared to normal human brain or cerebellum (Figure 2E).

In medulloblastoma (MB) cell lines, heterogeneous PI3KC2β expression was observed (Figure 2F). Microarry analysis of a panel of primary medulloblastoma samples also showed that PI3KC2β was over-expressed in 16/60 samples compared to normal human cerebellum (Supplemental Figure 1).

Together, these data revealed subgroups of tumours and cell lines displaying PI3KC2β over-expression, in which the class II PI3K may play a role in regulating proliferation, survival or migration.

Inhibition of cell proliferation by pharmacological PI3KC2β inhibitors or siRNA targeting PI3KC2β

To gain insight into the contribution of the class II PI3K isoform PI3KC2β in cell proliferation, two different isoform-specific pharmacological inhibitors (PI701 and PI702) were used. The specificity of these inhibitors was verified by *in vitro* PI3K assays using purified recombinant preparation of various PI3K isoforms. The IC_{50} values for specific inhibition of the enzymatic activity of PI3KC2β were 182 nM for PI701 and 652 nM for PI702 while values above 10μM were observed for the other PI3K isoforms (Table I). Both inhibitors also failed to inhibit the activity of mTOR and DNA-PK, with IC_{50} values above 100 μM (data not shown). A screen against a panel of 72 protein kinases *in vitro* confirmed that the two compounds are selective for PI3KC2β. Indeed, they only showed some inhibitory activity, at a concentration of 10 μM *in*

vitro, against anaplastic lymphoma kinase (ALK), c-Raf, p38-regulated/activated kinase (PRAK) and tyrosine protein kinase receptor B (TrkB)* (*PI702 only) (data not shown).

The anti-proliferative activity of both inhibitors was investigated in a panel of 28 cell lines and primary cultures from AML, GBM, MB, NB and SCLC. As controls, non-leukemic bone marrow cells (N), two immortalised B cells lines (FIN COS, 41b MI) and an immortalised Type II pneumocyte cell line (PN) were included. Both inhibitors of PI3KC2β inhibited cell proliferation in a dose-dependent manner with IC_{50} values below 10μM (PI701: 16/30 cases; PI702: 9/25 cases) (Figure 3 and Table II). The lowest IC_{50} values were observed in SCLC cell lines, AML blasts and D341 MB cells (Table II). In contrast, the IC_{50} values observed in control cells (FIN COS, 41b MI, N, PN) were above 10 μM. In several cases, cell lines with high PI3KC2β expression were more sensitive to PI701 and PI702, as was the case for AML (U937, NB4), MB (D341), GBM (T98G) and SCLC (H-209, H-510) (Figure 1 and 2, Table II).

Investigation of the effect of PI701 on activation of downstream signalling mediators in AML cell lines showed a dose-dependent decrease in the phosphorylation of key signalling molecules (Figure 4). These include Akt, c-Jun N-terminal kinases (JNK) and ribosomal S6 protein.

Together, these data show that inhibition of PI3KC2β with selective pharmacological inhibitors impairs proliferation in a subset of human cancer cells with IC_{50} values compatible with inhibition of the enzyme. This is in line with the finding that the phosphorylation of key signalling molecules is decreased upon treatment with PI701 in AML.

To validate the results obtained with pharmacological inhibitors, small interfering RNA (siRNA) targeting PI3KC2β was tested in a AML cell line. The specific down-regulation of PI3KC2β expression by siRNA was verified by Western blot analysis (Figure 5A). Decreased expression of PI3KC2β in U937 resulted in a 40% reduction of cell proliferation and was accompanied by increased caspase-3 activity (Figure 5B). The effects of the PI3KC2β siRNA on cell proliferation were less marked in THP1 cells, which displayed lower expression of the enzyme (data not shown).

Targeting PI3KC2β enhances the sensitivity of AML and GBM cells to chemotherapy

We next investigated whether inhibition of PI3KC2β by pharmacological inhibitors could modulate the sensitivity of AML and GBM cells to chemotherapeutic agents. In AML cell lines, combination treatment with PI701 led to markedly increased sensitivity to etoposide (Figure 6A). This effect was also observed in GBM cell lines and *ex vivo* cultures where PI701 combined with doxorubicin had a stronger effect on cell viability than the chemotherapeutic agent alone (Figure 6B and 6C). Moreover, the combination treatment enhanced the induction of apoptosis as assessed by increased caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage (Figure 6D and 6E).

In summary, these results highlight the importance of PI3KC2β in regulating the viability and chemoresistance of various tumour cells.

PI3KC2β contributes to cell migration in transformed epithelial cells

Previous reports have described a role for PI3KC2β in the migration of cancer cells (Katso et al., 2006; Maffucci et al., 2005). To investigate the specific contribution of PI3KC2β in the migration of tumourigenic vs. non-tumourigenic cells, murine epithelial cells sequentially transformed by oncogenic H-Ras and TGF-β1 were used (Maschler et al., 2005). To analyse the contribution of PI3KC2β to the migratory capacity of cancer cells, murine epithelial cells were treated with PI701. Western blot analysis revealed increased PI3KC2β expression in the FibRas cells, which have previously been shown to display enhanced migration and invasion, as compared to Eph4 and EpRas cells (Maschler et al., 2005) (Figure 7A). Treatment with PI701 impaired migration of FibRas cells but had no significant effect on the migration of Eph4 and EpRas cells (Figure 7B). At the concentrations used, PI701 did not significantly inhibit cell proliferation (data not shown). The finding that pharmacological inhibition of PI3KC2β impaired the migratory capacity of highly invasive cells emphasises the importance of PI3KC2β in regulating cancer cell migration.

DISCUSSION

To date, the class I_A isoform p110α is the only validated target of the PI3K family in the context of human cancer. Other class I_A isoforms have been associated with a role in various malignancies, including p110β in colon cancer, and p110δ in AML and breast cancer. Concerning the class II PI3Ks, PI3KC2α has been shown to play a role in survival of HeLa cells and increased expression of PI3KC2β was reported in a subset of tumours and cell lines from AML, GBM and SCLC (Arcaro et al., 2002; Knobbe & Reifenberger, 2003; Qian et al., 2002). Furthermore, PI3KC2β has been shown to play a role in the migration of A-431, HeLa and ovarian cancer cells, and to contribute to SCLC cell growth and Akt activation in response to growth factors such as SCF (Arcaro et al., 2002; Katso et al., 2006; Maffucci et al., 2005). In the present study, we have extended these studies on PI3KC2β in AML, brain tumours and neuroendocrine tumours.

Here we report that PI3KC2β is overexpressed in a variety of human cancer cell lines and primary cultures compared to control tissue. The growing interest in small molecule inhibitors has led to the development of a plethora of pharmacological PI3K inhibitors whose activity in human cancer remains to be validated. In the present study, the effect of two specific inhibitors of PI3KC2β (PI701, PI702) on cancer cell responses was investigated. Upon treatment with either compound a dose-dependent inhibition of cell proliferation was observed in various human tumour cells while non-tumourigenic cells such as immortalised B cells or type II pneumocytes remained largely unaffected. Moreover, a correlation between protein expression levels and sensitivity to the inhibitors was observed in a number of cell lines. In AML, siRNA against PI3KC2β also led to decreased proliferation accompanied by an increase in apoptosis. The finding that the activation status of important signalling molecules including Akt, JNK and S6K was reduced upon treatment with PI701 is in line with the inhibitory effect of this compound on cell proliferation.

Interestingly, cotreatment of cells with PI701 led to an increased sensitivity to chemotherapeutic agents such etoposide and doxorubicin. This supports previous reports showing that the inhibition of important survival pathways, including PI3K/Akt, can enhance the response to cytotoxic reagents (Abdul-Ghani et al., 2006; Kumar et al., 2005). In the present study we have demonstrated for the first time that PI3KC2β inhibitors can sensitise human cancer cells to chemotherapeutic agents such as etoposide and doxorubicin.

Besides its role in regulating cell proliferation, in invasive murine breast cancer cell lines PI3KC2β was also found to be involved in cell migration. Enhanced expression of PI3KC2β was detected in the most motile cells (FibRas) as compared to non-invasive cells (EpH4). Treatment of these cells with increasing doses of PI701 strongly reduced the migratory capacity in wound healing assays. This is an interesting observation considering that malignant cells often acquire the ability to migrate leading to the propagation of metastases throughout the body.

In summary, the present study illustrates that PI3KC2β plays a crucial role in regulating various cellular responses in a broad spectrum of human cancer cells. Since the pharmacological inhibitor PI701 not only reduced basal cell proliferation but also had an effect on chemosensitivity and cellular migration, PI3KC2β could prove to be an attractive target for cancer treatment in the future.

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FIGURE LEGENDS

Figure 1 PI3KC2β expression in AML and SCLC cells. Western blot analysis of PI3KC2β expression in (**A**) normal bone marrow cells (N), primary blasts (1-7), AML cell lines and immortalized B cells (FIN COS, 41b MI) and (**B**) type II pneumocytes (PN) and SCLC cell lines (left panel). Relative mRNA expression levels of *PIK3C2B* in (**A**) human AML cell lines and (**B**) SCLC cell lines (right panel). Nontransformed type II human pneumocytes were used as a control.

Figure 2 PI3KC2β expression in tumours of the central nervous system. In (**A-B**) neuroblastoma cell lines and (**C-D**) primary tumour samples, PI3KC2β expression was analysed both by Western blot analysis (A, C) as well as quantitative RT-PCR (B, D). PI3KC2β protein expression in (**E**) glioblastoma cell lines and *ex vivo* cultures and (**F**) medulloblastoma cell lines by Western blot analysis. For neuroblastoma samples, human adrenal tissue (AG) was used as a control. For glioblastoma and medulloblastoma samples, normal human cerebellum served as a control.

Figure 3 Titration curve of PI701 in AML and SCLC. (**A**) AML cell lines (U937, triangles; HL-60, circles) and patient blasts (FAB M1, asterisks) and (**B**) SCLC cell lines (H209, triangles; H510, circles) were treated with increasing concentration of the inhibitor for 72 hours. For (**A**) AML cell lines and blast cells, immortalised B cells (diamonds and squares) served as a control. For (**B**) SCLC, nontransformed type II pneumocytes (squares) were used as a control.

Figure 4 Effect of PI701 on the phosphorylation of downstream signalling molecules. AML cell lines (U937 and THP1) were treated with increasing concentrations of PI701 overnight.

Pathway activation was visualised by monitoring the phosphorylation status of Akt (P-AktSer^{473}), JNK (P-JNK$^{Thr183/Tyr185}$) and S6 protein (P-S6 protein$^{Ser235/236}$).

Figure 5 Inhibition of AML cell proliferation by small interfering RNA (siRNA) targeting PI3KC2β. (**A**) U937 cells transfected with control siRNA or siRNA targeting PI3KC2β were analyzed by western blotting for protein expression. (**B**) Cell proliferation of U937 cells transfected with siRNA targeting PI3KC2β was analysed by MTS assay (left panel) and caspase-3 activity was assessed in parallel. **P<0.01 by analysis of variance test.

Figure 6 PI701 sensitises AML and glioblastoma cells to chemotherapeutical agents. (**A**) Titration curve of U937 (left panel) or THP1 (right panel) cells incubated with increasing concentrations of etoposide in the absence (black circles) or presence (white circles) of PI701 (1μM). (**B**) Titration curve of T98G (left panel) or U251 (right panel) cells incubated with increasing concentrations of doxorubicin in the absence (black circles) or presence (white circles) of PI701 (1μM). (**C**) Titration curve of two glioblastoma *ex vivo* cultures incubated with increasing concentrations of doxorubicin in the absence (black circles) or presence (white circles) of PI701 (1μM). A representative experiment (out of three) performed with eight repetitions is shown for A-C. *P<0.05 or **P<0.01 by analysis of variance test. (**D**) T98G cells were treated with PI701 or doxorubicin alone or in combination and caspase-3 cleavage was evaluated by means of a Western blot. (**E**) U937 cells were treated with increasing concentration of etoposide either alone or in combination with PI701 and PARP cleavage was visualised by means of a Western blot. The band corresponding to cleaved PARP (85 kDa) is shown.

Figure 7 Role of PI3KC2β expression in mammary epithelial cell lines. (**A**) Western blot analysis of PI3KC2β expression in EpH4, EpRas and FibRas cells. (**B**) Effect of PI701 on migration of mammary epithelial cell lines. Cells were treated with increasing concentrations of PI701 and the distance migrated was measured after 8 hours.

Supplemental Figure 1 Microarray analysis of PI3KC2β expression in a panel of primary medulloblastoma samples and cell lines. Gene expression profiles were obtained by using the Affymetrix HG-U133 Plus 2.0 array.

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Table I IC_{50} values (μM) against isolated enzymes.

Compound	PI3KC2β	p110α	p110β	p110γ	p110δ
PI701	**0.528**	**> 10**	**> 10**	**> 10**	**> 10**
PI702	**0.632**	**> 10**	**> 10**	**> 10**	**> 10**

Table II IC_{50} values (μM) of PI701 and PI702 against a panel of cancer cell lines and primary cultures from NB, GBM, MB, AML, and SCLC.

IC50 Values		
	PI701	**PI702**
Neuroblastoma Cell Lines		
CHP134	7.4	8.0
LAN1	8.3	10.0
SHSY5Y	15.8	6.6
WAC2	5.2	6.0
Glioblastoma Cell Lines and *ex vivo* Cultures		
T98G	6.9	> 20
U251	8.0	> 20
LN319	19.6	14
LN229	6.2	14.1
U87	> 20	10.2
LO	11.6	ND
RE	13.2	ND
SB	18.2	ND
Medulloblastoma Cell Lines		
DAOY	10.1	> 20
D341	4.5	4.0
AML Cell Lines, Patient Blasts and Control Cells		
U937	3.2	5.5
HL-60	9.6	12.4
NB4	5.3	7.8
THP1	8.4	9.5
Kasumi	> 20	ND
KG-1	17.0	8.0
K562	10.4	> 20
FIN COS	> 20	> 20
41b MI	> 20	> 20
FAB M5	2.5	ND
FAB M1	3.6	ND
FAB M0	ND	6.2
N	ND	12.6
SCLC Cell Lines and Control Cells		
H69	4.4	ND
H209	4.3	ND
H510	5.0	ND
SW2	10.2	ND
PN	12.0	ND

LO, RE, SB = glioblastoma *ex vivo* cultures; FIN COS, 41b MI = immortalised B cells; FAB = French-American-British classification; N = non-leukemic bone marrow cells; PN = immortalised Type II pneumocytes; ND = not determined

Figure 1

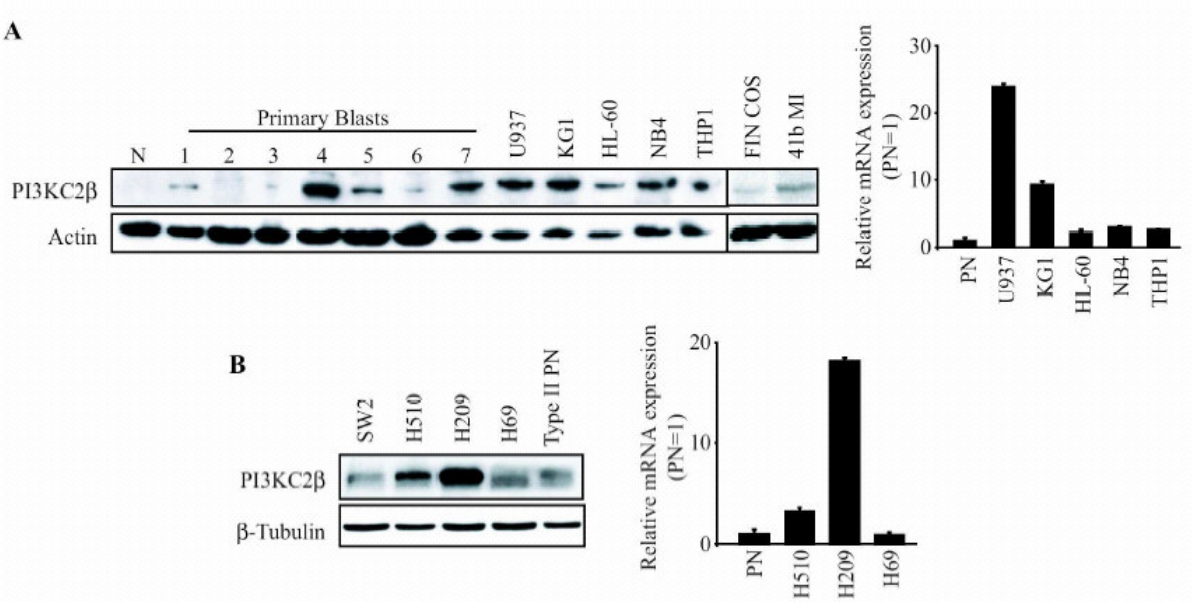

A
Primary Blasts
N
1
2
3
4
5
6
7
U937
KG1
HL-60
NB4
THP1
FIN COS
41b MI
PI3KC2β
Actin
Relative mRNA expression (PN=1)
30
20
10
0
PN
U937
KG1
HL-60
NB4
THP1
B
SW2
H510
H209
H69
Type II PN
PI3KC2β
β-Tubulin
Relative mRNA expression (PN=1)
20
10
0
PN
H510
H209
H69

Figure 2

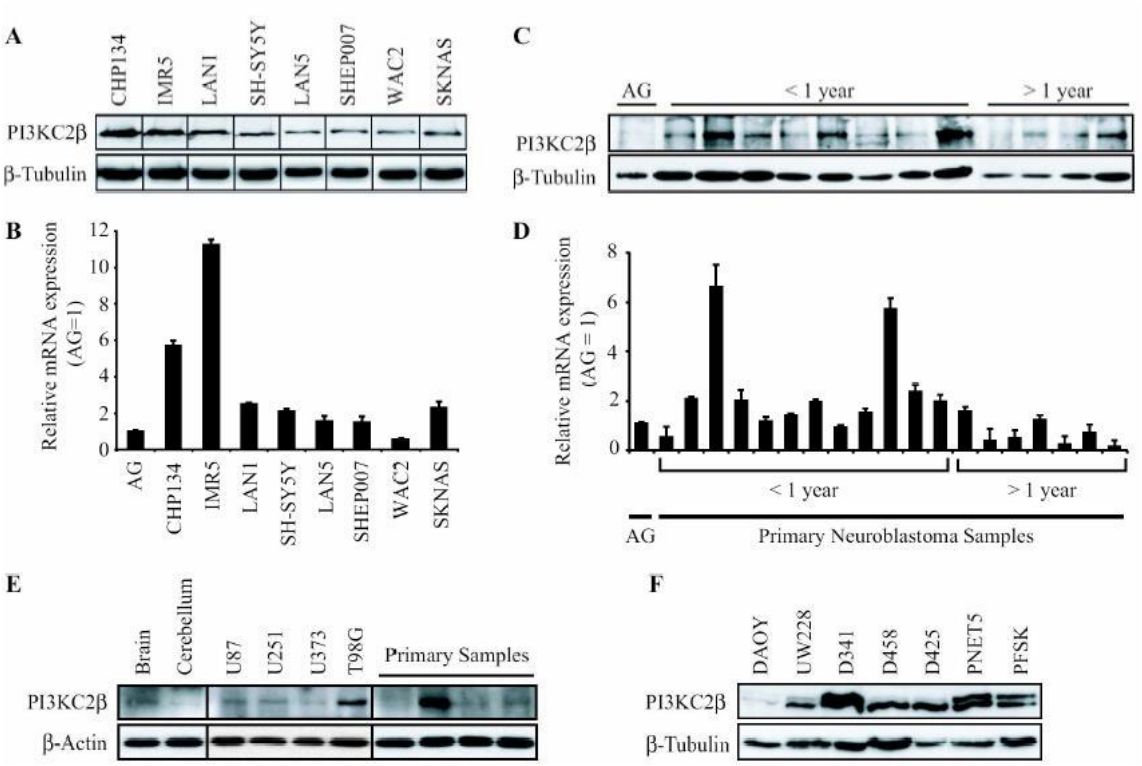

A
CHP134
IMR5
LAN1
SH-SY5Y
LAN5
SHEP007
WAC2
SKNAS
PI3KC2β
β-Tubulin
C
AG
< 1 year
> 1 year
PI3KC2β
β-Tubulin
B
Relative mRNA expression (AG=1)
12
10
8
6
4
2
0
AG
CHP134
IMR5
LAN1
SH-SY5Y
LAN5
SHEP007
WAC2
SKNAS
D
Relative mRNA expression (AG = 1)
8
6
4
2
0
< 1 year
> 1 year
AG
Primary Neuroblastoma Samples
E
Brain
Cerebellum
U87
U251
U373
T98G
Primary Samples
PI3KC2β
β-Actin
F
DAOY
UW228
D341
D458
D425
PNET5
PFSK
PI3KC2β
β-Tubulin

Figure 3

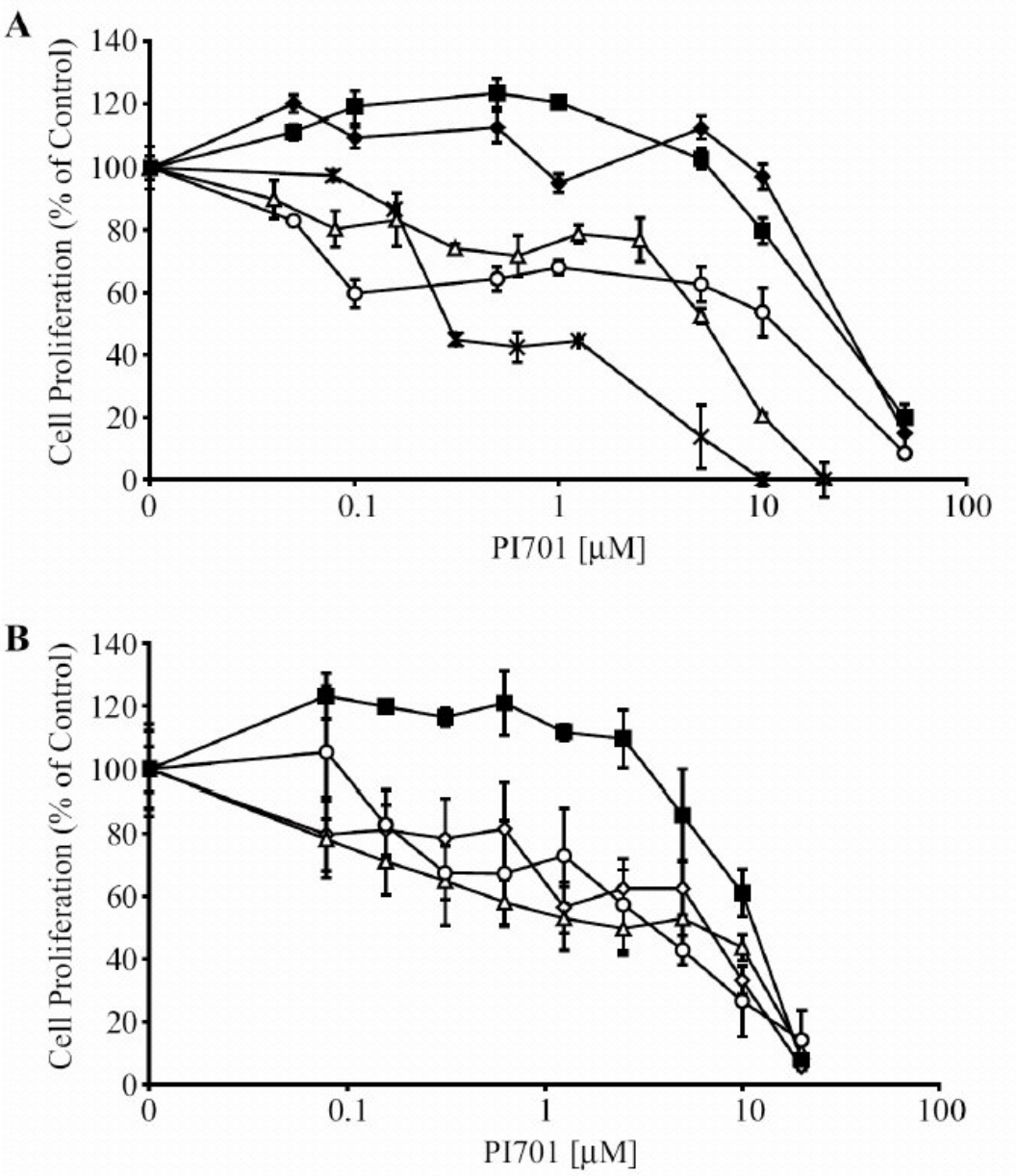

A
Cell Proliferation (% of Control)
140
120
100
80
60
40
20
0
0
0.1
1
10
100
PI701 [µM]
B
Cell Proliferation (% of Control)
140
120
100
80
60
40
20
0
0
0.1
1
10
100
PI701 [µM]

Figure 4

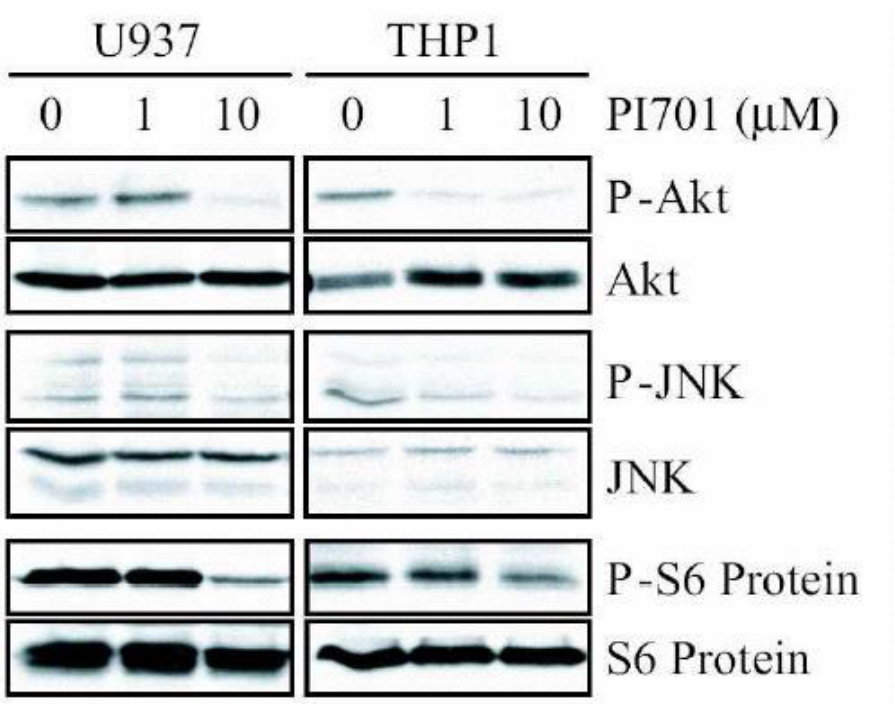

U937
THP1
0
1
10
0
1
10
PI701 (μM)
P-Akt
Akt
P-JNK
JNK
P-S6 Protein
S6 Protein

Figure 5

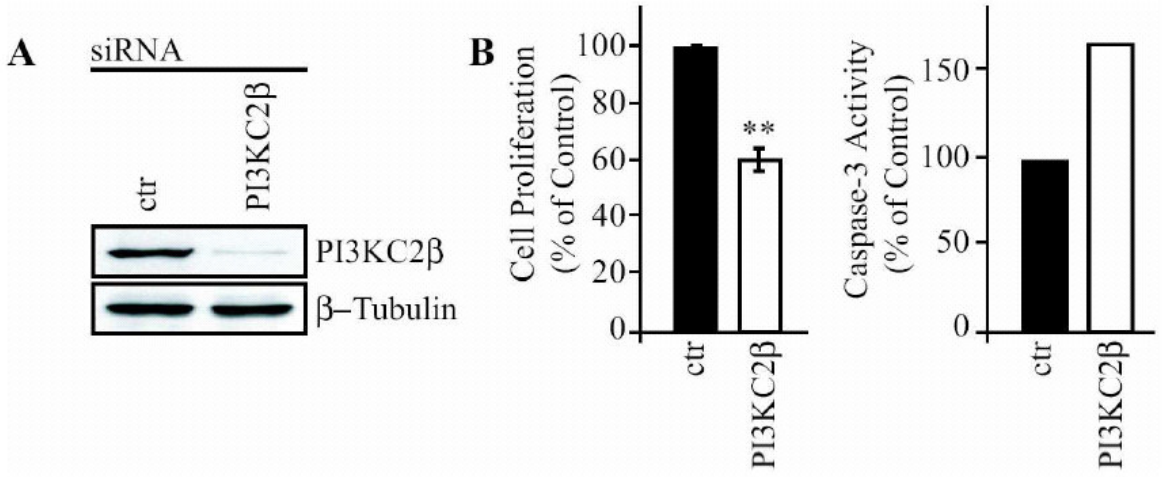

A
siRNA
ctr
PI3KC2β
PI3KC2β
β–Tubulin
B
Cell Proliferation (% of Control)
100
80
60
40
20
0
**
ctr
PI3KC2β
Caspase-3 Activity (% of Control)
150
100
50
0
ctr
PI3KC2β

Figure 6

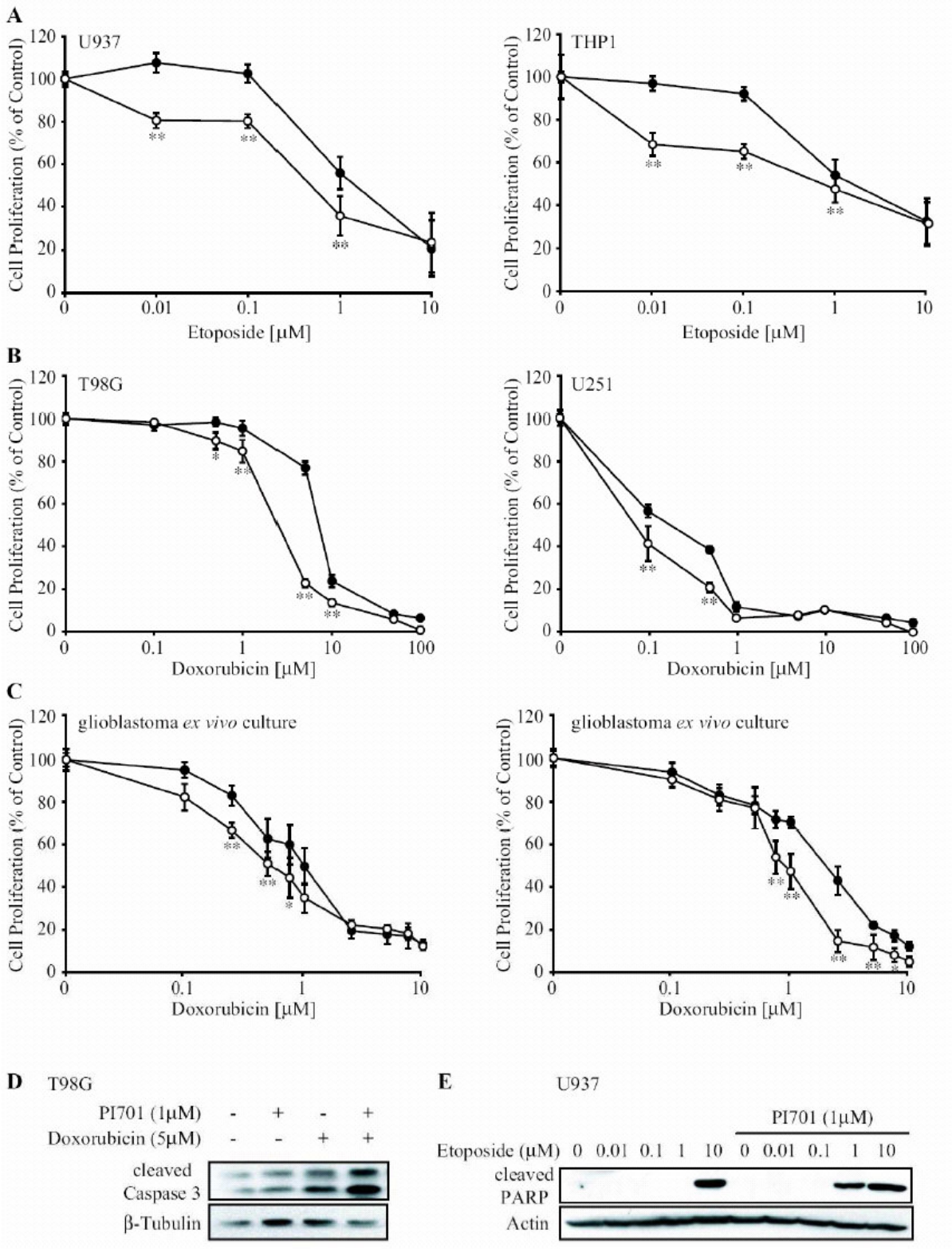
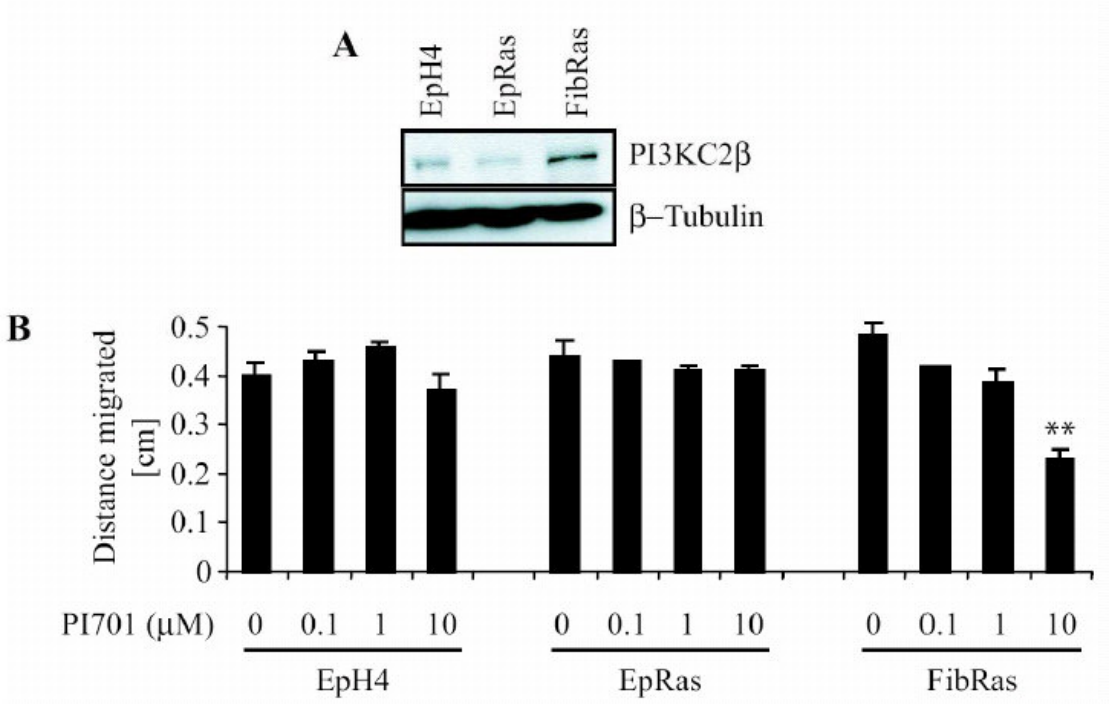
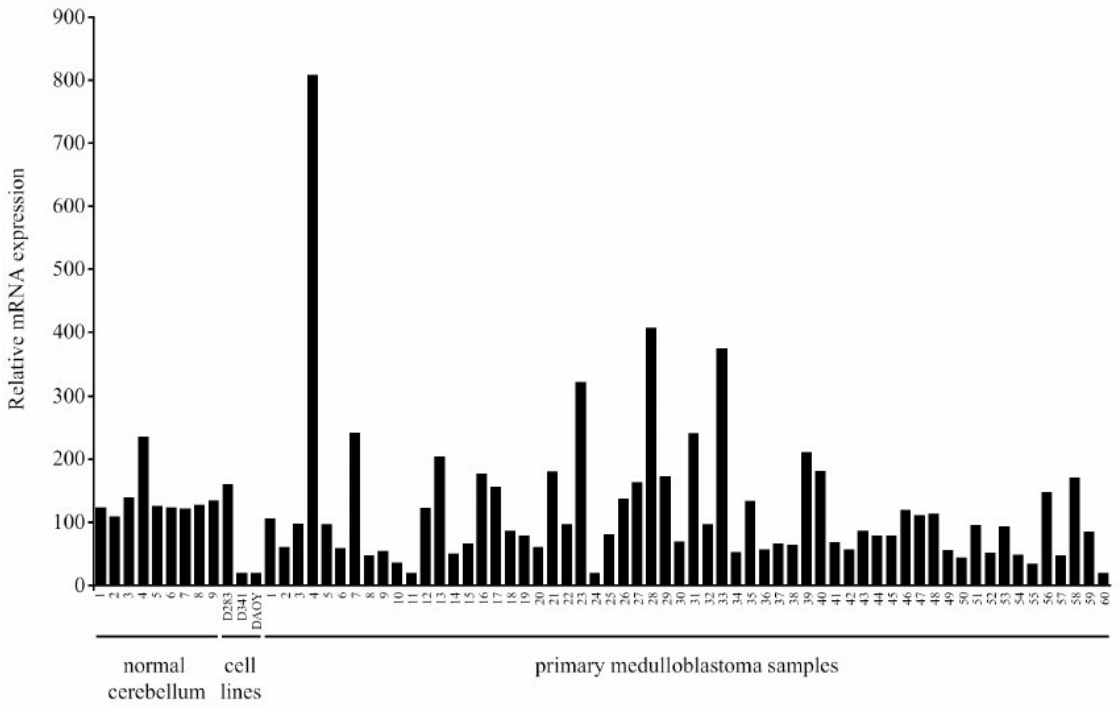

A
U937
THP1
Cell Proliferation (% of Control)
Etoposide [µM]
B
T98G
U251
Doxorubicin [µM]
C
glioblastoma ex vivo culture
glioblastoma ex vivo culture
D
T98G
PI701 (1µM)
Doxorubicin (5µM)
cleaved Caspase 3
β-Tubulin
E
U937
PI701 (1µM)
Etoposide (µM) 0 0.01 0.1 1 10 0 0.01 0.1 1 10
cleaved PARP
Actin

Figure 7


A
EpH4
EpRas
FibRas
PI3KC2β
β–Tubulin
B
Distance migrated [cm]
0.5
0.4
0.3
0.2
0.1
0
**
PI701 (μM)
0 0.1 1 10
0 0.1 1 10
0 0.1 1 10
EpH4
EpRas
FibRas

Supplemental Figure 1



3.2.4 Investigation of the role of Src family Tyrosine Kinase in the regulation of PI3KC2□

3.2.4.1 Abstract

PI3KC2□ is a class II PI3-kinase family member able to phosphorylate the D3 hydroxyl group of a downstream target phosphoinositide. Several important pathways involve PI3KC2□ that once phosphorylated and thereby activated, transduce the signal, inducing different cell processes like proliferation, migration and actin rearrangement. From the sequence of this protein, 4 tyrosines were identified as possible site of phosphorylation (Y68, Y127, Y228, Y1541). Our previous studies proved a crucial role for Src proteins in activating PI3K class I members suggesting a possible second role for Src in regulating also Class II members.

On the base of this observation we used HEK293 cells and proved that Src was able to interact with PI3KC2□ increasing substantially its lipid kinase activity. With migration and proliferation tests, we then identified a crucial involvement of Src and PI3KC2□ in inducing the response in HEK293 cells. We went on investigating which of the possible phosphorylation sites are important for PI3KC2□ activation. We finally concluded that Src activates PI3KC2□, but when tyrosines 68 and 127 in the NH_2 terminal domain of PI3KC2□ are mutated, the effect of Src on PI3KC2□ activation was abolished in migration and proliferation assays. F-actin staining also confirmed that the mutants carrying phenylalanine instead of these two tyrosines were not able to induce the cytoskeletal organization that was evident in cells transfected with Src and PI3KC2□ wild type.

3.2.4.2 Introduction

PI3Ks are a family of enzymes able to phosphorylate the D3 hydroxyl group of the inositol ring in phosphoinositides. The substrate can be PtdIns or all the other phosphoinositides, already phosphorylated on the other hydroxyl group. These kinases are subdivided into three different classes (I-III). Class I are the most studied members and regulate several cell functions like cell proliferation, cytoskeletal structure, protein synthesis and migration [84, 160, 300]. Recent observations also identified class II members as playing a crucial role in cell functions, but there are still few reports [84, 301-303].

Class II PI3Ks were first identified by sequence homology to other PI3Ks [304, 305]. The three members of class II (PI3K-C2□, PI3K-C2□ and PI3K-C2□) are monomers of high molecular weight, 170 KD [165]. The characteristic of this class of PI3K is the presence of two C2 domains, rather than only one as in the class I PI3K members. The second carboxy-terminal C2 domain present in these proteins possesses different properties from the C2 domain of the other PI3Ks. Differently from class I, these PI3Ks are composed by only one subunit and they do not seem to bind any regulatory subunit. Phosphorylation of different tyrosines, may control the activation of this enzyme, but nothing is demonstrated yet. Several possible sites of phosphorylation are present in the N-terminal and in the C2-terminal domain of all members, but which one is really important is still unknown [306]. Deletion of C2 domain in fact, increase lipid kinase activity suggesting that it also act as a negative regulator of the catalytic domain [84].

Molecular interactions by which extracellular stimuli are linked to class II of PI3Ks are unclear at the present. Some results suggest that the RTKs are able to activate PI3K Class II members [84]. In this paper we show that Src is able to activate PI3KC2□. We demonstrate that the two proteins are able to interact and that Src phosphorylates PI3KC2□ increasing its kinase activity. We also went further identify a possible role for Src and PI3KC2□□in inducing proliferation and migration.

3.2.4.3 Materials and Methods

Antibody and reagents—All the antibodies and the vectors were chosen and utilized according to[155]

Transient expression in Mammalian cells-- HEK293 cells were grown to 50-60% confluence on 100mm dishes and transfected with cDNA constructs in pcDNA3 using calcium-phosphate protocol as already described [307]. The cells were harvested 24h post transfection and analysis for gene expression, protein kinase activity and plated for cell migration and proliferation.

Immunoprecipitation--Cells grown on 150mm dishes were placed on ice and then lysed and immunoprecipitated as described in [308].

SDS-PAGE and Western blot analysis--Cells cultivated in 10 cm-dishes were lysed in lysis buffer, and SDS-PAGE and Western blot was performed as previously described [308]. The gels were transferred onto hydrophobic polyvinylidene difluoride membrane (Hybond-P; Amersham Bioscience). This membrane was than blotted with different antibodies. For detection, the enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences) were used according to the manufacturer's protocol.

PI3KC2 -Kinase Assays-- PI 3-kinase activity of the immunoprecipitates was assayed in according to [308]

Cell Migration, Wound healing assays--Cells were platted in 12-wells culture plates in complete culture medium in a way to be confluent after 24h. At that time a wound was created using a disposable 200 µl micropipette tip in order to scrape off the cells. Pictures were then taken every 30 min for 8 h using a Leica9 DM IRBE Inverse, wide field Microscope (EMZ, University of Zurich) in order to follow the wound closure. In the case of transfected cells the transfection was performed 48h in advance and cells were plated 24h before the wound healing assay.

Cell Motility, time lapse assays--Cells were platted 24h before the experiment, in a 12-well plates in a way to be 30-40% confluent the day after. Cell migration was then monitored by taking pictures every 10 min for 12h. The program ImageJ was used to make a video. Single cells were followed and the speed of cells was measured under the different experimental conditions.

Cell Proliferation

Cells were plated at two different concentration 5x103/well and 10x103/well in 96-well plates and left to growth respectively 8h and 48h in complete DMEM. For every condition 8 wells were plated. The number of viable cells was then measured by means of an MTS assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Immunofluorescence for F-actin- Cells were seeded on glass coverslips and let grown for 24 h. In the case of inhibitors, the cells were then treated for 3 days. Then cells fixed in methanol, and permeabilized in 0,1% Triton X-100 were incubated with rhodamine-conjugated phalloidin to detect F-actin. Nuclear staining was perfomed by incubating cells with DAPI before coverslip mounting. The coverslips were then washed in PBS and incubated in fresh Triton X-100 for 10-15 min in order to permeabilise the cells. After washing again in PBS1x an anti-actin antibody was added and after 20min was added also phalloidin and left for an additional 30 min. Coverslips were then washed in PBS, incubated with DAPI for 10 min and washed again PBS and finally fixed by Movieo on the cover slide.

3.2.4.4 Results

Src protein phosphorylates and associates with PI3KC2□ via its kinase domain.

Most of the knowledge regarding PI3K family of proteins is concerning class I members and less is known regarding class II proteins. In recent year a rapid increase in knowledge about cell signaling, drove our attention on the possible crucial role of Src protein in phosphorylating and activating PI3KC2□ kinase [309]. In order to investigate whether Src is able to direct interact with PI3KC2□ inducing its phosphorylation and activation, HEK293 cells were transfected with Src and PI3KC2□□□myc tagged, separately or in combination (Fig. 1). 24h after transfection cells were lysed and immunoprecipitated respectively for Src or myc. A Western blot analysis was then performed, revealing that in both the cases there is a coimmunoprecipitation of the Src-PI3KC2□ complex indicating the interaction of the two proteins in cells. Further analysis regarding the phosphorylation state of the two proteins revealed that in the presence of co-expression PI3KC2□ was phosphorylated. When C2□ is expressed alone or co-expressed together with Src kinase dead it was not phosphorylated anymore but is still present in the immunoprecipitation. Together this results clearly indicate that the two proteins interact *in cells* and that the phosphorylation by Src is possible only throw its catalytic activity.

In vitro PI3KC2□ activation by Src

Since Src and PI3KC2□ are able to interact *in cells* leading to the activation of the second protein we were interested in understanding if the two proteins interact directly and if Src directly phosphorylate PI3KC2□ without involving other proteins.

To better answer to this question, the phosphorylation assay was performed also *in vitro*.

PI3KC2□ protein was added in two different concentrations in buffer solutions containing only Src or ATP or both together and then incubated at 37° (Fig. 2A). From a subsequent Western blot analysis, tyrosine phosphorylation of PI3KC2□ by Src was observed, the signal was clearly stronger where higher concentration of PI3KC2□ was present (Fig.2A). Phosphorylation of PI3KC2□ was not present anymore in the conditions where or ATP or Src were absent. This result shows that Src is able to bind directly PI3KC2□ thereby inducing its phosphorylation. To examine the impact of Src-mediated tyrosine phosphorylation of PI3KC2□ on its lipid kinase activity *in vitro*, a kinase assay was performed (Fig.2B).

PI3KC2□ was pre-incubated with Src and ATP and a kinase assay was subsequently performed. When Src previously phosphorylated PI3KC2□ an increase in the PI(3)P formation was present. Where the kinase wasn't previously activated there is almost no presence of PI(3)P. This showed that pre-phosphorylation by Src induces a significant increase in the lipid kinase activity of PI3KC2□.

Src-PI3KC2□ complex activation increase cell migration and cell proliferation.

Very often, PI3KC2□ is active in cancer cells, suggesting a possible role for this protein in inducing these characteristics. Already in human epithelial carcinoma cell line (A431) stimulated with EGF we demonstrated a crucial involvement of PI3KC2□ in inducing cell proliferation and migration [84]. We thereby proposed that kinase activation can be induced by Src. To prove this hypothesis HEK293 cells were transfected with Src and PI3KC2□ separately or in combination and we investigated possible effects of these proteins interaction on migration (Wound Healing and Random Migration) and proliferation (MTS). The wound healing speed calculated, revealed a significant increase in migration in the cells transfected with both the proteins, while cells presenting the expression of only one of the two are not affected (Fig.3A). In order to confirm this data, increase in cell migration ability was also checked by random migration, confirming what the results observed in the wound healing (Fig3B). Part of transfected cells where plated for an MTS assay that also showed a relevant rise in proliferation of cells transfected with Src and PI3KC2□ together. This results clearly confirm that the role played by Src and PI3C2□ is crucial in cell for migration and proliferation.

PI3KC2□ mutants in tyrosine phosphorylation sites are not functional.

On the base of these evidences we wanted to elucidate which tyrosine is phosphorylated by Src inducing PI3KC2□ activation. On the base of the phosphorylated sites described in this protein by Cell signaling, we created different mutants each of them carrying a single or combinations of mutations located respectively in the NH_2 terminal domain or in the C2 terminal domain (fig.4). As already mentioned before there are some specific tyrosines which are possible site of phosphorylation. Some evidences in tumors suggested that there are four specific tyrosine that can be phosphorylated in PI3KC2□. These amino acids are respectively

located: three in the NH_3 terminal domain (Y68-Y127-Y228) and one in the C2 terminal domain (Y1541) (Fig.4). We then obtained several plasmids coding for PI3KC2□ with single mutations or carrying two ore more mutations in different combinations. We already clearly demonstrated that when in HEK293 cells, functional PI3KC2□ is activated together with Src, cells acquire the ability to proliferate and migrate more (Fig3 A-B). Thereby the mutants were tested in comparison with the wt and we transfected them in 293 cells together with Src. The results (Fig.5A) indicate that some of the mutants (2-3-6-7-9) were not affecting cell migration like PI3KC2□ wt. The MTS assay (Fig 5B), identify the same mutants to be not functional also in inducing proliferation. Together these results suggest that Mut2 (Y127F), Mut3 (Y228F), Mut6 (Y127F/Y228F), Mut7 (Y68F/Y127F/Y228) and Mut9 (Y68F/Y127F/Y1541) abrogate Src-mediated activation of PI3KC2□.

F-Actin staining confirms that Mut 2/3/6/7/9 are not active.

After the results obtained in migration and proliferation assay, we focalized our attention on five of these mutants that seems to be inactive. Our interest was to investigate whether differences in migration reflected differences in cytoskeletal organization.

In order to answer to this question, Mut2/3/6/7/9 plasmids were cotransfected with Src in HEK293 cells and after 24h an immunostaining for F-actin was performed.

Normal cell shape appear generally rounded or just a bit elongated (Fig. 6A). The absence of actin accumulation around the borders reflected the absence of lamellipodia and ruffles, which confirmed the low migration capacity of these cells.

After cotransfection of Src and PI3KC2□ in cells, the morphology changed drastically. The form of the cells presented long cytoplasmic elongations and irregular shape, similar to fibroblast cells (Fig.6B). Actin accumulation along the membrane demonstrated formation of ruffles and filopodia, typically present in migrating cells. In the pictures from cells transfected with the selected mutants (Fig. 6C), it was evident that there is no change in cell morphology when compared to wt cells and that there is almost no evidence of actin accumulation around the surface of the cell. These data confirm previous results regarding the inactivity of these 5 mutants (Fig. 6C). They confirm that PI3KC2□ together with Src induce changes in cell shape, contributing to increase in cell motility.

3.2.4.5 Discussion

PI3K protein members have been identify as key members of most of cell function like survival, proliferation, migration and gene expression regulation [310, 311].

Class I members have been long investigated since considered crucial for most cell types, but subsequently, Class II captured the attention of researchers too.

Class II PI3K consists of three distinct isotypes: C2□, C2□ and the last to be described and predominantly expressed in liver C2□. Differently from class I, these isoforms consist of only one subunit containing all the domains, for protein binding, for the catalytic activity and the COOH- terminal C2 domain [165].

The function of this last domain in PI3KC2□ is still unknown but despite that it gives the name to the protein family, since it seems to have special characteristics in comparison with the C2 domain also present in the other PI3K members. Recent observations demonstrate that deletion of the C2 domain increased the *in vitro* lipid kinase activity towards PI, suggesting that this domain may, therefore compete with the kinase domain for binding the PI substrate [308]. This evidence suggest that the C2 domain has a negative regulation on the lipid kinase activity of PI3KC2□, but how the two domains work and are coordinated.

For class I PI3K it has been described that Src kinases interact with p85 regulatory subunit inducing activation of the lipid kinase activity [312].

On the base of this, a possible role for Src in inducing also class II PI3K activation was suggested. In our *in vitro* and *in vivo* experiments it was clear that Src is really able to interact and directly phosphorylate PI3KC2□ and by the kinase assay we also showed that phosphorylation by Src was increasing its activity.

The roles of PI3KC2□ in cells are not fully understood and therefore we investigated if its activation could be important in migration and proliferation. We show here that co-expression of Src and C2□ in cells, are essential for both these cell abilities. Migration and proliferation are extremely essential for both, normal and cancer cells, and the pathway responsible for them in not fully understood. Most is known regarding cell migration, suggest a crucial role for class I PI3K, but our results clearly provide evidences for an essential role also for PI3KC2□. From studies based on the protein sequence, conformation and identified phosphorylations we selected four tyrosine as possible target for Src phosphorylation.

These tyrosines are located respectively: three in the NH_2 terminal domain and one in the COOH- terminal C2 domain. We thereby created several mutants and after migration and

proliferation studies, we hypothesized that mutants carrying Y68 and Y127 mutations present an ineffective activation by Src. This evidence kept our attention on these two tyrosine even if, some mutants carrying them together with other mutations, do not show any reduction in inducing migration and proliferation. We hypothesized that probably other mutation can restore PI3KC2□ activity.

This model is still under investigation and further experiments have to be performed in order to clarify which tyrosine is involved and is essential in PI3KC2□ activity. Since PI3KC2□ appears to be involved in migration, and since migration clearly requires actin conformation changes, we performed other studies on actin remodeling which confirmed that these mutants are not functional.

3.2.4.6 Figures

Figure 1

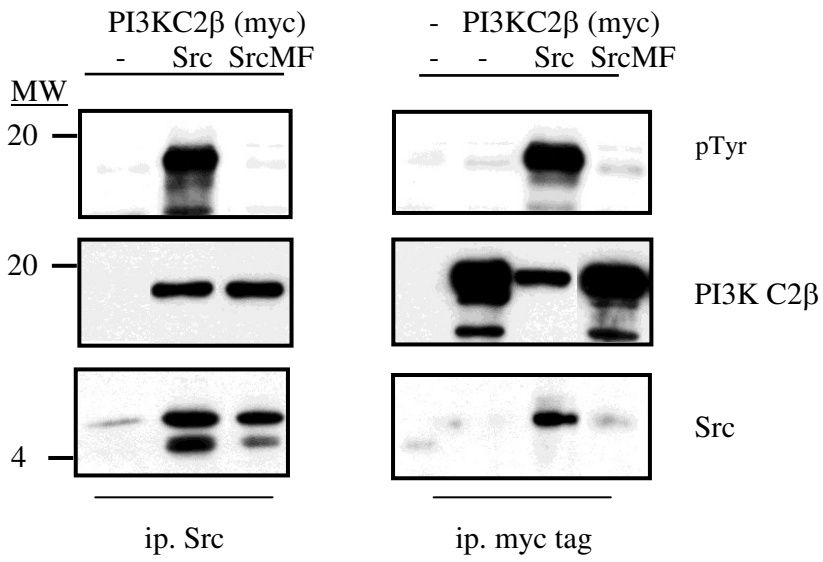


Figure 1. ***In cell*** **PI3KC2β phosphorylation assay**.
HEK293 cells were transfected with PI3KC2β alone or together with wt or kinase inactive Src. After 24h cells were lysed and immunoprecipitation for myc or Src was performed and then Western blot analysis was done.

Figure 2

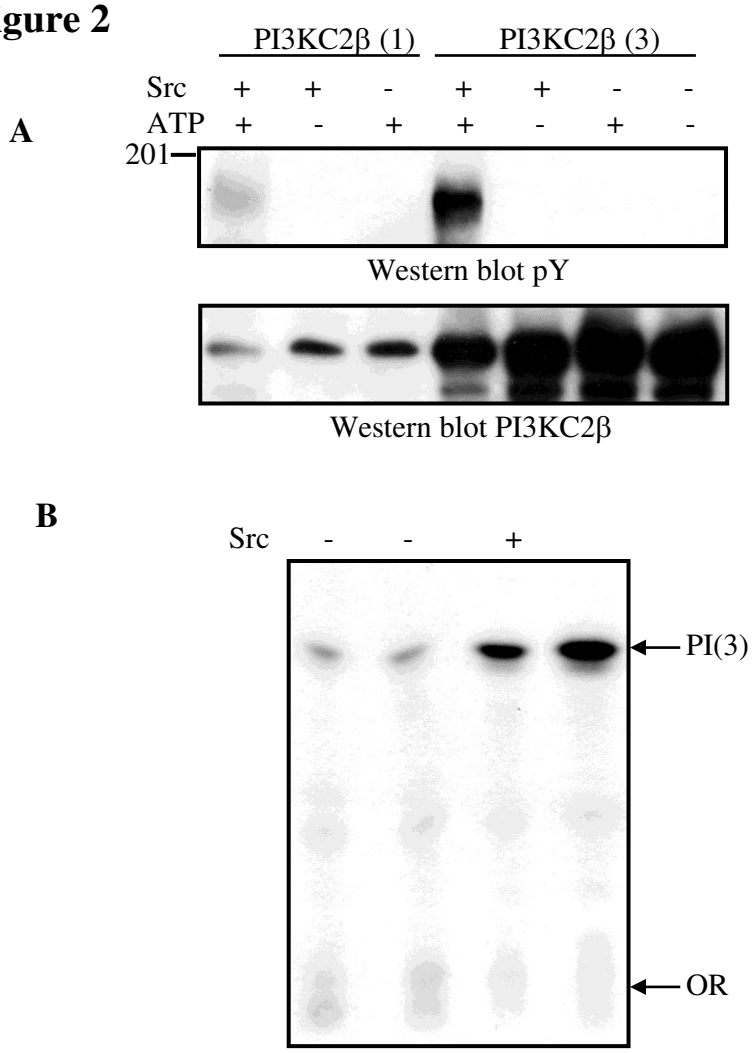


Figure 2. in vitro phosphorylation and kinase assay.
PI3KC2β protein was mixed together with Src and ATP in combination, or separated. After an incubation time, Western blot analysis was performed (A). A solution containing PI3KC2β, PI and radioactive ATP was prepared. After 5 min of incubation a TLC was run and the PI(3)P presence was investigated (B).

Figure 3

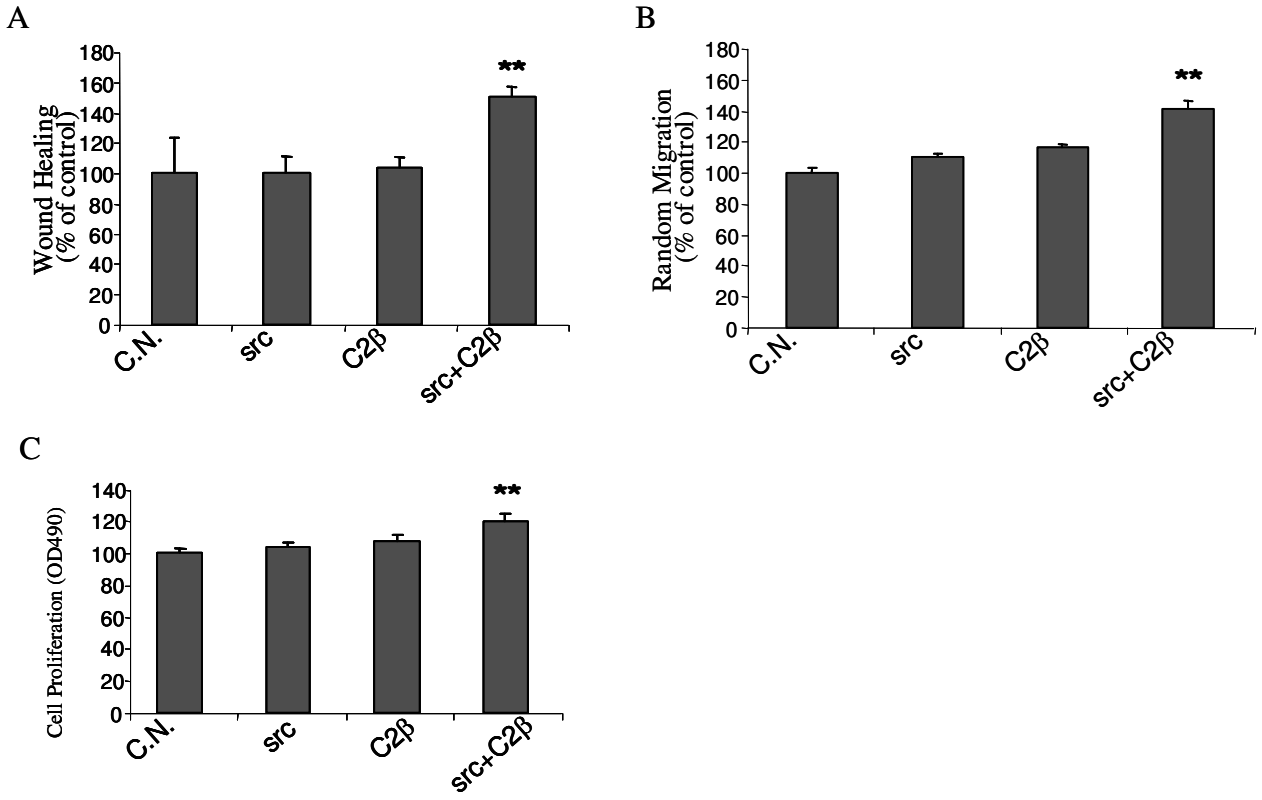


Figure 3. **Cell migration and proliferation analysis**.
HEK293 cells wt or transfected with Src alone or in combination with PI3KC2β were analysed for migration by wound healing assay (A) or by random migration (B). Part of the transfected cells were plated for proliferation assay, MTS assay

Figure 4

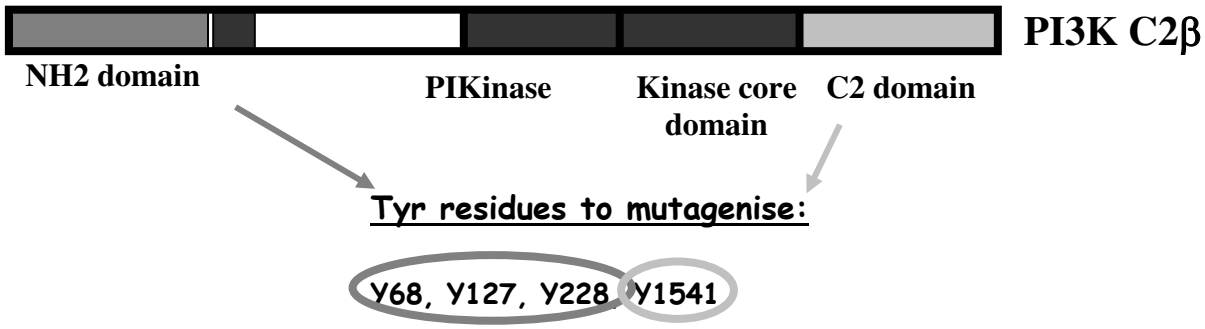


Figure 4. PI3KC2β protein structure and mutants list.
PI3KC2β contains several domains and the 4 most common phosphorylation sites in this protein are located in the NH_2-terminal domain and in the C2 domain. PI3KC2β mutants were created, containing one or more Tyrosine residues mutated to phenylalanine.

Figure 5

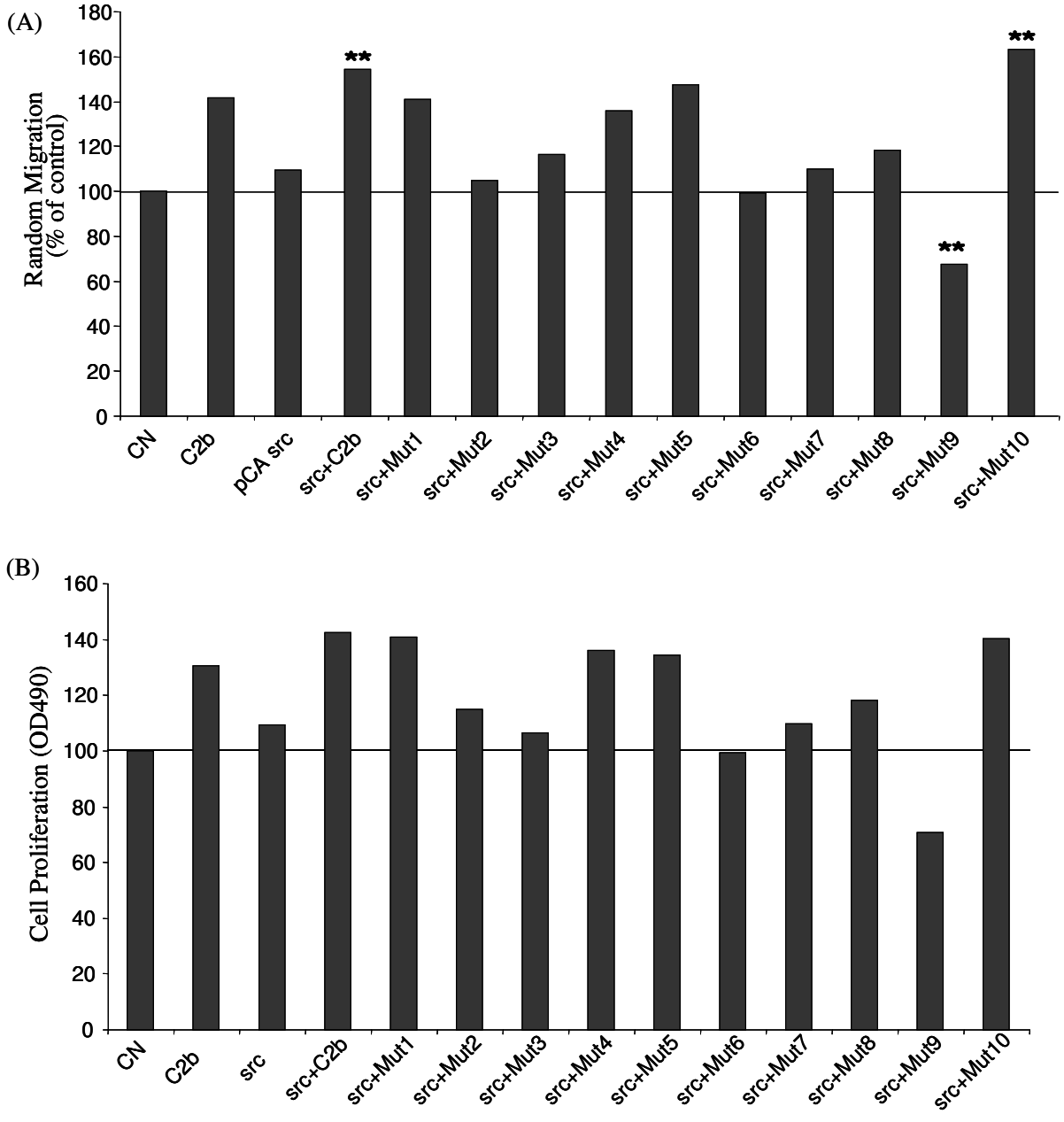


Figure 5. Mutants analysis in HEK293 cells.
Cells were transfected with Src in combination with PI3KC2□ mutants. Part of transfected cells was used for Random Migration assay (A). The rest of the cells were platted for an MTS analysis.

Figure 6

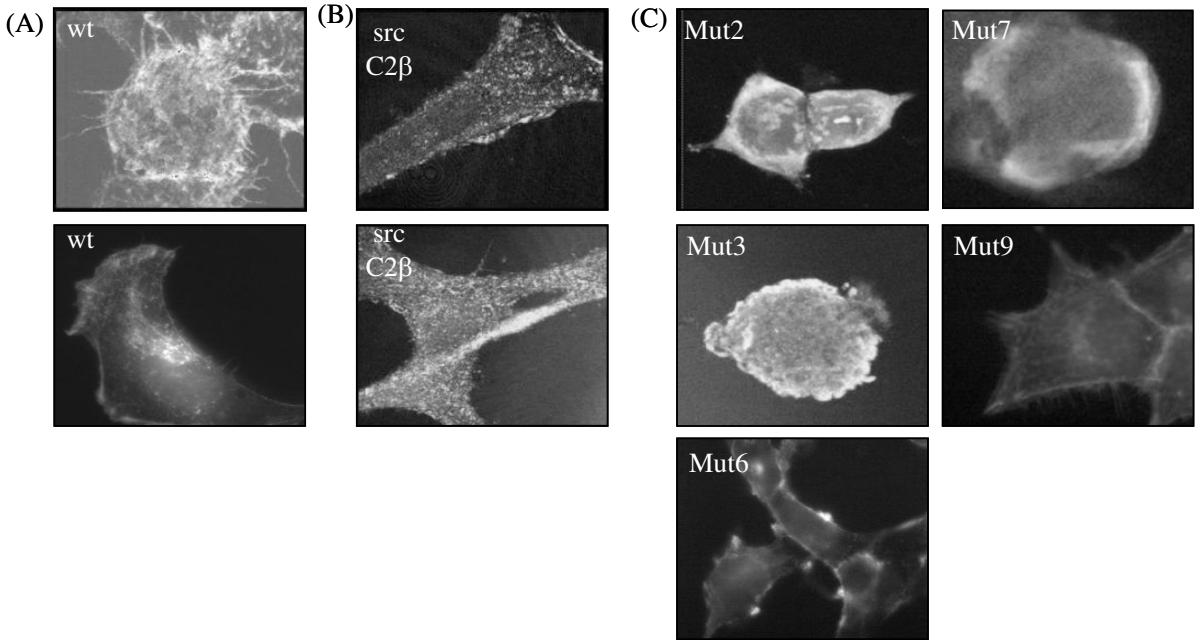


Figure 6. Actin and PI3KC2□ location analysis.
Transfected cells with Src, PI3KC2□, Mut 1-10 in different combination were plated and stained with fluorescent phalloidin (F-actin) (A,B,C). In this figure are shown the mutants inducing the most evident effect on F-actin organization.

4 Discussion

The aim of this study was to investigate the role of Ras and TGF□ in breast cancer.

More than 95% of breast cancers develop from the epithelial cells, arising from either the milk producing glands (lobular carcinoma) or the draining ducts (ductal carcinoma) [5]. On the base of this evidence three different cell types were systematically compared (EpH4, EpRas, FibRas). They offered a new and attractive model able to mimic the three state of metastatic breast cancer development: epithelial cells (EpH4), oncogenic Ras-transduced epithelial cells (EpRas) and metastatic cells that already underwent the EMT transformation (FibRas) [313]. EpRas cells are epithelial cells transfected with the active form of the H-Ras gene. In fact it has already been shown that Ras play a crucial role in tumor formation [110]. It is now commonly accepted that epithelial cells over-expressing H-Ras protein generate human breast cancer cells [314]. Furthermore it was demonstrated that TGF□ is a growth factor present in most breast cancer and that when added to cells already presenting H-Ras over-expression, it is able to increase cell migration and invasion [315, 316]. It was proved that these cells after treatment with TGF□ undergo the EMT transformation characteristic of metastatic cells [317]. This model offers a novel approach for investigate the pathway involving TGF□ and Ras in breast cancer and metastasis development. The use of pharmacological inhibitors in migration and proliferation experiments, revealed that the MAPK pathway was mainly involved in proliferation, rather then PI3Ks that revealed to play a crucial role in migration and invasion. Based on this result, the main focus of this project was to investigate the contribution of the individual PI3K isoforms in FibRas cells migration. The idea that the members of the PI3K superfamily have different roles in cell functions is already widely accepted. The data presented in this dissertation are in agreement with this idea and we further investigated it. We in fact demonstrated for the first time that class I_A PI3K is crucial in FibRas signaling inducing cell migration, proliferation and actin rearrangements. In fact inhibition of p110□ or p110□ function by using pharmacological isoform-specific PI3K inhibitors or by short hairpin RNA (shRNA)-mediated silencing drastically impaired migration of FibRas cells. These class I_A PI3Ks have been already suggested to contribute to the pathophygiology of ovarian cancer and also contribute to cell anchorage-independent growth [318, 319]. *PI3KCA* has been found mutated at high rate in

breast cancers [216, 217]. Despite all these observations, nothing is known about p110□□specific pathway activation and all the data reported until now regard in general all PI3Ks members. p110□□ has been also proved as able to induce p53 activation and since this p53 is a crucial and amply studied oncogene, this gave much more emphasis to the possibility of a key role for PI3K class I_A in cancer [318] . On the contrary, regarding p110□ no specific roles have been described so far.

Having demonstrated that PI3K class I_A and class I_B are crucial in migration and proliferation of breast cancer, we considered the subset of proteins that could have been probable targets of *PI3KCA* and *PI3KCB*. We demonstrated that the activity of S6K, cdc42 and Rac was drastically reduced after inhibition of p110□ and p110□. We also showed that these three proteins drastically affect migration. These observations together proved for the first time that p110□ and p110□ directly activated S6K, cdc42 and Rac and that this pathway induces breast cancer cell migration. The fact that they induce migration was not surprising since especially for cdc42 and Rac it is commonly known that these proteins are involved in actin rearrangements and thereby in affecting cell tumor migration [320-322]. Regarding their functions in breast cancer less is known but their crucial role seems to be accepted [323]. The literature reporting about S6K and migration is really scarce but this enzyme may also be important in cell migration [324], although it has a different role from actin reorganization [325]. Together with these observations is known that S6K, cdc42 and Rac are downstream targets of PI3Ks [200, 326].

Together with p110□ and p110□ there is a third catalytic subunit in class I_A PI3K, namely p110□. The role of p110□ appeared to be antagonist of p110□ and p110□. In fact its expression was detectable only in EpH4 and not in EpRas and FibRas cells. We demonstrated that when restoring its expression in EpRas and FibRas, cellular migration was drastically impaired. These results were quite surprising, since they are in contrast with some other studies already published [327]. However, reports about p110□ in cancer cell migration are few and ours is the first regarding specifically breast cancer.

The Rho protein is largely accepted to be crucial in increasing cell adhesion, thereby in inhibiting migration [328]. In accordance with this widely accepted model, we confirmed that its overexpression reduces breast cancer cells migration. This suggests that Rho could definitely be a possible target downstream of p110□. Even if nothing is known regarding this

specificity for p110□, Rho is generally considered a downstream protein of the PI3K pathway [329]. Here for the first time we evidenciated a selective regulation of p110□ and Rho, in fact inhibition of p110□ and p110□ did not impair Rho activity. We also found that p110□ expression induces AKT activation. Therefore we decided to test the ability of AKT to impair cell migration in breast cancer. These results evidenced a negative impact on migration. AKT is an already well known protein activated by PI3K [330]. The last result confirmed the role of p110□ in inducing Rho and AKT activation and reducing migration. Here for the first time we classified this role as being specific for p110□ isoform. This study reports novel findings in the field of class I PI3K isoforms and their signaling pathway activation. From a clinical point of view these findings offer knowledge for developing new anticancer treatments that would be more specific, thereby more efficient and presenting less side effects.

In other studies, we focalized our attention on the class II PI3KC2□ isoform. This protein is a member of the class II PI3Ks and comparing to the class I PI3K, very little is known about it. PI3KC2□ together with PI3KC2□ have been shown to be targets of the EGFR in human carcinoma-derived A431 cells. In fact after stimulation of these cells with EGF the activity of PI3KC2□ was significantly increased [331]. About the mechanism of this activation nothing was proved yet. Many models already demonstrated to be valid for class I activation by the EGFR could be valid also for class II PI3K members. In fact it was already demonstrated that the EGFR is able to recruit the p85 regulatory subunit to the membrane and thereby activate PI3K catalytic activity [332]. Different models were suggested for this recruitment. It was already demonstrated that this RTK is able to directly recruit and bind p85 through its SH2 domain [152]. But since this domain is missing in class II PI3K, this model was not applicable. it was then discovered that the EGFR also recruits some adaptor proteins and that through these it can then recruit p85. The adaptor protein suggested for this role are the Abi1/Grb2/Eps8 complex [332, 333]. Following this observation we tested the possible involvement of this complex also in activating PI3KC2□. We could demonstrate that after cell stimulation with EGF, PI3KC2□ was detected in the immunoprecipitated fraction of the Eps8/Abi1/Sos complex. This clearly proved the novel idea that EGFR recruit PI3KC2□ by employing the adaptor complex Eps8/Abi1/Sos. In the literature, other adaptor proteins like Grb2 and Shc have been described in addition to this complex. Because of this we also investigated their possible involvement in our model. What we observed is that also Grb2 and

Shc can form a complex with PI3KC2β and induce its activation. We then further demonstrated for the first time that in human carcinoma cell lines Shc/Grb2/PI3KC2β and PI3KC2β/Eps8/Abi1/Sos1 complexes, are in reality only one macromolecular complex and not two. We also proved that this complex is able to transducer the EGFR signal, down to the PI3KC2β. Going on investigating possible downstream targets of PI3KC2β we could clearly show that after EGF stimulation also the activity of Rac was increased. This activity of Rac resulted in upregulation of cell migration. This last evidence was in reality expected, since Rac activation induced by PI3K class I has been largely described. In addition, the crucial role played in migration by Rac is already accepted [321]. In this report, we also proved for the first time that PI3KC2β recruitment also renders A-431 cells resistant to anoikis. Not much is known about Rac and anoikis, but what is described until now suggest the possibility that resistance to anoikis can be induced by two pathways, one involving Rac and another independent from this protein. This evidence was reported in epithelial cells, therefore our results are the first experiments in carcinoma cell lines [334].

An additional study on PI3KC2β revealed that this protein also contributes to the regulation of proliferation and survival in several tumors. In fact the expression pattern and functions of the PI3KC2β isoform were investigated in a panel of tumour samples and cell lines. Overexpression of PI3KC2β was found in subsets of tumours and cell lines from acute myeloid leukemia (AML), glioblastoma multiforme (GBM), medulloblastoma (MB), neuroblastoma (NB), and small cell lung cancer (SCLC). The treatment of these tumor samples and cell lines with pharmacological inhibitors for PI3KC2β (PI701 and PI702) led to a dose-dependent inhibition of proliferation. The same results were obtained silencing PI3K C2β by siRNA. These effects of PI3KC2β on cellular responses were tested in a large number of cancer cell lines and ex-vivo cultures. These studies clearly demonstrated that this protein plays a crucial role in several cell activities, such as proliferation and migration. These properties were critically impared when together with the PI3KC2β pharmacological inhibitor, a chemotherapeutic agent, like doxorubicin, was added. Our recent results in HEK293 cells also proved that PI3KC2β activation can be achieved through Src protein recruitment. The ability of Src to activate PI3Ks is already known, even if, also in this case, all the observations concern class I PI3K members. The activation of PI3KC2β through Src

increase migration and proliferation of HEK293 cells. Furder experiments are now going on in order to identify which phosphorylation site in PI3KC2β is crucial for its activation.

In summary, all these studies demonstrate that all the components of PI3K class I can regulate different functions in cancer cells. This offers new knowledge about PI3K signaling and could offer new ideas for developing novel therapeutic strategies, more specific and thereby with less side effects. In fact, one of the most important problems in breast cancer is that many treatments used until now have very strong and delayed side effects, which are much worse than their advantages in breast cancer treatment.

All the results concerning PI3KC2β roles and tissue-specific distribution, taken together can be fundamental for a better understanding tumor progression, offering then, again a new opportunity for developing isoforms-specific PI3K inhibitors hopefully usable in new therapies.

5 Review articles

5.1 Lipid Rafts and Caveolae in Signaling by Growth Factor Receptors

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Lipid Rafts and Caveolae in Signaling by Growth Factor Receptors

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Abstract: Lipid rafts and caveolae are microdomains of the plasma membrane enriched in sphingolipids and cholesterol, and hence are less fluid than the remainder of the membrane. Caveolae have an invaginated structure, while lipid rafts are flat regions of the membrane. The two types of microdomains have different protein compositions (growth factor receptors and their downstream molecules) suggesting that lipid rafts and caveolae have a role in the regulation of signaling by these receptors. The purpose of this review is to discuss this model, and the implications that it might have regarding a potential role for lipid rafts and caveolae in human cancer. Particular attention will be paid to the epidermal growth factor receptor, for which the largest amount of information is available. It has been proposed that caveolins act as tumor suppressors. The role of lipid rafts is less clear, but they seem to be capable of acting as 'signaling platforms', in which signal initiation and propagation can occur efficiently.

INTRODUCTION

The 'fluid mosaic' model envisions the plasma membrane to contain proteins floating freely in a uniform 'sea' of lipid. However, the plasma membrane is no longer thought of as a uniform structure, following the identification of microdomains known as lipid rafts and caveolae. These microdomains are of a less fluid character than the remainder of the membrane, and are enriched in certain proteins, in particular those involved in signal transduction. This has led to the hypothesis that they might act as membrane signalling platforms. The aim of this article is to discuss the possible role that these membrane microdomains play in growth factor receptor signal transduction. We will therefore begin by describing the structure of lipid rafts and caveolae and the proteins that are targeted to these domains, before going on to discuss in detail their possible roles in the signalling of certain growth factor receptors. Lastly, we will discuss how the alteration of signalling in these microdomains may lead to cancer in humans.

CAVEOLAE

Caveolae were first described in the 1950s as 50-100 nm flask-shaped invaginations of the plasma membrane [1] (Fig. **1A**). Caveolae have been found to be insoluble in non-ionic detergents such as Triton X-100, and this property has been used to purify and characterise these domains. Following treatment with detergent at 4ºC, these insoluble domains are then separated from the remainder of the cell membrane by ultracentrifugation in a sucrose density gradient [2]. Alternatively, a detergent-free fractionation procedure can be used, following which caveolae are present in low buoyant density fractions [3]. The insolubility of caveolae in detergent is thought to be due to an enrichment in sphingolipids and cholesterol [4, 5], as opposed to the majority of the cell membrane, which is composed mainly of glycerophospholipids. Unsaturated acyl chains are common in the latter, while sphingolipids contain largely saturated chains, allowing them to pack together more tightly. Hence, glycerophospholipids exist in a loosely packed, liquid-disordered phase (the liquid crystalline, l_c phase). In the absence of cholesterol, sphingolipids form a very solid phase known as the gel phase; however, in caveolae cholesterol is present, and this causes these domains to exist in a new phase, the liquid-ordered phase (l_o) [6]. In this phase the lipids are still tightly packed, but also have a high degree of lateral mobility. These properties confer insolubility in non-ionic detergents, and depletion of these domains of either sphingolipids or cholesterol increases their solubility in Triton X-100 [7].

Caveolae possess a striated coat on their cytoplasmic face, major components of which are proteins known as caveolins. There are now three known members of the caveolin gene family: caveolin-1 (two isoforms α and β) [8, 9], caveolin-2 (three isoforms α, β and δ) [10] and caveolin-3 [11]. The N- and C-termini of caveolins are cytoplasmic, suggesting that they form a 'hairpin' structure in the membrane (Fig. **1A**), and they are palmitoylated on several residues [12]. Caveolins –1 and –2 have a wide, overlapping distribution: they (and therefore caveolae) are present in most cell types, being most abundant in endothelial cells, fibroblasts, adipocytes, pneumocytes and epithelial cells [13, 14]. In contrast, caveolin-3 is almost solely expressed in smooth and skeletal muscle [15].

Two forms of caveolae, 'deep' and 'shallow', have been identified, with different distribution of the three caveolins and the respective isoforms [10, 16]. It is possible that these domains have functional differences, but this has not been sufficiently studied. Caveolins are thought to be an important structural component of caveolae, which is probably related

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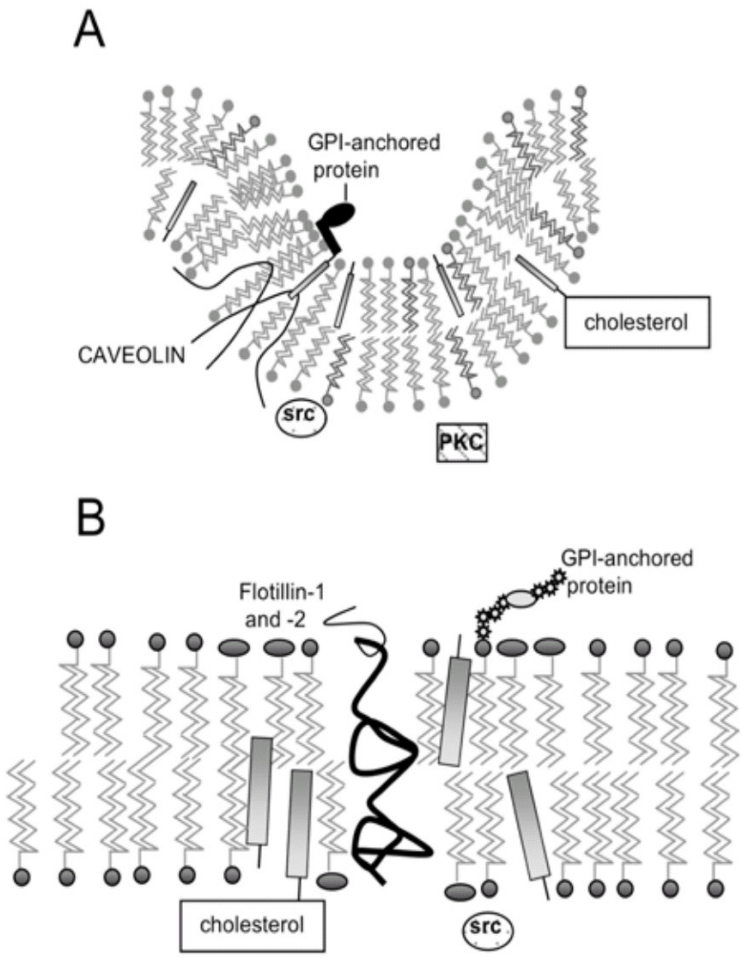

Fig. (1). Structure of lipid rafts and caveolae. A) Caveolae; B) Lipid Rafts. Caveolin molecules in (**A**) are shown as hairpin-shaped structures in the inner leaflet; N- and C-termini project into the cytoplasm. GPI-anchored proteins are anchored on the outer leaflet of caveolae and lipid rafts, while signalling molecules such as Src kinase associate with the inner leaflet.

to the ability of caveolin to bind cholesterol [17] and sphingolipids [18]. More precisely caveolin-1 or caveolin-3 re-expression in cells lacking both caveolae and caveolin expression induces caveolae formation and reconstitutes the caveolar coat [19]. However, expression of caveolin-2 alone doesn't lead to caveolae formation, but requires co-transfection of caveolin-1 [19, 20] Caveolins are phosphorylated on Ser/Thr sites by PKCα and on Tyr by Src and possibly other tyrosine kinases [21].

The number of caveolae in a cell and the level of caveolin expression are also intimately linked to the concentration of cholesterol. Increasing the level of free cholesterol in fibroblasts by increasing the external concentration of low density lipoprotein (LDL) led to an upregulation of caveolin mRNA and increased the number of caveolae [22], while depleting cholesterol using cholesterol-sequestering agents led to a decrease in caveolin mRNA levels [23]. In turn, decreasing caveolin concentration lowered the rate of cholesterol efflux from the cells, suggesting that caveolae are involved in cholesterol homeostasis [22].

In addition to caveolins, another family of proteins has been shown to form an integral component of caveolae. These are the flotillins, flotillin-1 and flotillin-2 (epidermal surface antigen) [24]. Flotillins can form hetero-oligomeric complexes with caveolins, and are probably structural components, which participate in the formation of caveolae [25].

LIPID RAFTS

The discovery that detergent-insoluble domains were present in cells lacking caveolae and caveolins [26, 27] suggested that membrane microdomains distinct from caveolae might exist. These domains can also co-exist with caveolae in the same cell, and can be isolated separately [28] and are present in the same membrane fractions as caveolin, using detergent-dependent or –independent methods. They have been coined 'lipid rafts' [29], and have an estimated diameter of less than 70 nm [30], possibly being as little as 26+/-13nm [31]. Like caveolae, they are enriched in spingolipids and cholesterol, but do not seem to form any particular structure as caveolae do, rather being flat regions of the cell membrane that exist in the liquid ordered phase (Fig. **1B**).

Unlike caveolae, they are probably found ubiquitously, while caveolae are excluded from certain cells, such as T lymphocytes [32].

There have been concerns that lipid rafts might be artefacts arising from the treatment of membranes with detergent at low temperature [33]. Firstly, treatment with Triton X-100 is usually performed at 4°C, and the cooling of the membrane by itself promotes the formation of the liquid ordered phase [6]. In addition, Triton X-100 was shown to promote the formation of liquid-ordered domains when added to an originally homogeneous membrane [34]. Despite these observations, there is evidence that lipid rafts exist in the liquid ordered phase prior to addition of detergent. Ahmed *et al.* [5] used a detergent-free, fluorescence quenching technique to show that phase separation did occur in membranes with a similar composition to the plasma membrane, suggesting the co-existence of a liquid-ordered phase containing sphingolipids and cholesterol, and a liquid crystalline phase. Phase separation occurred at 37°C, and was promoted by cholesterol. Furthermore, the degree of insolubility in Triton X-100 correlated with the proportion of the membrane that was in the liquid-ordered phase. This suggests that these domains are probably present before detergent extraction; however it must still be noted that detergent probably alters the properties of these domains and their interaction with certain proteins, and that results obtained following treatment of membranes with detergent must be treated with caution. The detergent method certainly cannot be used to determine the difference between lipid rafts and caveolae when both these domains are present in the same cell.

Other lines of evidence also support the existence of lipid rafts in cell membranes [35]. For example, Varma and Mayor [30] used fluorescence resonance energy transfer (FRET) to show that glycosylphosphatidylinositol (GPI)-anchored folate receptors attached to a fluorescent folic acid analogue were clustered in domains of a size less than 70 nm. Depleting the cell membrane of cholesterol resulted in a decreased FRET efficiency, consistent with an increased distance between folate receptors. Addition of cholesterol to the cell resulted in restoration of the proteins to ordered domains. These results have been taken as proof of the existence of cholesterol-rich microdomains into which these proteins cluster. This hypothesis is further supported by work by Friedrichson and Kurzchalia [36], who used chemical cross-linking to demonstrate that GPI-anchored proteins were clustered at the plasma membrane, and that depletion of cholesterol decreased clustering, whereas addition of cholesterol increased the size of the clusters. The strongest evidence for non-caveolae nanodomains (50–200 nm in dimension in the outer leaflet of the plasma membrane) comes from immunogold electron microscopy of components of, for example, the T cell receptor (TCR) in fixed cells, where labelled proteins are detected in clusters [35].

Since lipid rafts are small and transient, the biophysical challenges of measuring them are still great. Indeed, the most controversial area of membrane lateral organization is on the nanoscale level, where technology with sufficient simultaneous spatial and temporal resolution is not yet available. Refining existing methods and developing new ones to study lipid rafts is now required for progress, with the goal of studying dynamic membrane structure in living cells [35]. The applicable techniques, for the most part, rely on fluorescence microscopy because of high sensitivity and applicability to single, living cells. Several techniques with the potential to most directly detect and characterize lipid rafts in living cells were reviewed recently [35].

Interestingly, it has been shown that flotillin-1 and flotillin-2 (also known as reggie-2 and –1) are localised in lipid rafts [37] and co-localise with GPI-anchored cell adhesion molecules, known to be found in rafts. It is necessary to note here that, although the outer leaflet of lipid rafts (Fig. **1**) is thought to be composed of sphingolipids and cholesterol, the nature of the inner leaflet in unknown.

PROTEINS ASSOCIATED WITH LIPID RAFTS AND CAVEOLAE

Besides the caveolins and flotillins, numerous other proteins have been associated with lipid rafts and caveolae. As mentioned above, there is evidence for the clustering of GPI-anchored proteins in lipid rafts, and they have also been identified in caveolae [38]. The existence of different functional GPI anchors as well as the fact that different rafts show markedly different lipid and protein profiles implies the presence of heterogeneous group of anchors and corresponding rafts [39, 40].

In addition, many proteins associated with the microdomains are receptors or proteins involved in signal transduction. These include G protein α-subunits, which have been shown to bind to caveolin *in vitro* [41]. A later study showed that different G protein subtypes localised preferentially to particular microdomains [42]. Several G-protein-coupled receptors have also been localised to membrane microdomains; some of these only become localised to these domains upon activation – for example the muscarinic acetyl choline receptor in caveolae [43]. Interestingly, a downstream component of signalling by this receptor, endothelial nitric oxide synthase, is localised to caveolae, and is thought to be negatively regulated by caveolin [44].

A number of other proteins that are able to interact with caveolin localise to caveolae. A detergent-free method, which gives a more accurate view of the proteins associated with rafts/caveolae than the detergent method [45], was used to demonstrate the co-fractionation of caveolin with H-Ras [46], c-Src, cdc42, Rho, Lyn and Fyn [47]. Ras was found to interact with caveolin, although a mutationally activated form of Ras would not bind to caveolin [48]. Furthermore, another study showed that caveolin binds to c-Src, although it will not bind mutationally-activated v-Src [49]. In addition, binding of caveolin-1 or caveolin-3 inhibited the autophosphorylation and activation of c-Src and the related Src-family tyrosine kinase Fyn [49]. These interactions were mediated by residues 82-101 in the cytoplasmic N-terminus of caveolin-1, a region of the protein which is called the 'scaffolding domain', and which interacts with numerous proteins. Furthermore, it has been described that caveolin phosphorylated on Tyr 14 can inhibit Src through the recruitment of c-terminal src kinase (Csk). The scaffolding domain of caveolin-1 seems also to be responsable for further interactions, since it contains a motif conserved in guanine nucleotide dissociation inhibitors (GDIs) which suggested a novel role for caveolin-1. Nevins and Thurmond demonstrated the interaction between caveolin-1 and Cdc42

[50]. Further studies also revealed a role in the activation and regulation of Rho, suggesting a role of caveolin-1 in cell polarity and migration [51]. del Pozo *et al.* [52] reported that lipid rafts are involved in signal transduction events initiated by cell adhesion to the extracellular matrix, which is mediated by integrins. They reported that lipid rafts are controlled by integrins to target Rho and Rac GTPases to specific plasma membrane domains and to couple them to their downstream effector molecules [53-55].

A detergent-free method was used to demonstrate the localisation of a number of proteins including Ras, the adapter protein Grb2, Erk2 (extracellular signal-regulated kinase 2) and the tyrosine kinases Fyn, TrkB (tyrosine kinase receptor B) and nerve growth factor (NGF) receptor (TrkA) in low buoyant density domains lacking caveolin-1 [56]. Lipid rafts have also been associated with signalling molecules in lymphocytes, which do not contain caveolin. For example, the T-cell receptor has been located in detergent-insoluble rafts following its activation, as have the signalling molecules Shc, Ras, Syk, and the Src kinases Lck and Fyn [32, 57, 58]. It must however be noted that these two studies utilised the detergent extraction method, and it has been shown that proteins associated with rafts and caveolae can be differentially soluble in different detergents [59], possibly due to a difference in their interactions with rafts/caveolae or due to the presence of distinct types of raft [60]. Therefore the detergent extraction method does not give an accurate view of all the proteins present in rafts. Finally, recent studies suggest that the differential localization of rafts is important in the control of cell migration [61, 62].

Table **1** summarises the proteins and lipid species found in lipid rafts and caveolae. This is not an all inclusive list but contains many of the proteins and lipids relevant to this discussion. What causes these proteins to target to lipid rafts and caveolae? Melkonian *et al.* [63] hypothesised that this might depend on the lipid modification of the proteins and their affinity for the liquid-ordered phase. Acyl chains, such as myristate and palmitate, and those found in GPI-anchored proteins, are saturated and likely to partition into liquid-ordered domains, while, for example, prenyl chains are bulkier and less likely to favour this phase. Melkonian *et al.* [63] showed that detergent-insoluble membranes were indeed enriched in palmitoylated proteins, although not all palmitoylated proteins in the cell were targeted to these domains. In contrast, prenylated proteins were excluded from the domains. In a later study, Zacharias *et al.* [64] produced similar results using a detergent-free method. They used FRET between two variants of green fluorescent protein: cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), which had been mutated so that they were unable to dimerize. Attaching myristoyl and palmitoyl chains to CFP and YFP caused them to form clusters in the membrane, and this was disrupted by depleting cholesterol. In addition, they were shown to cluster with caveolin. However, while prenylated fluorescent proteins were seen to cluster, this was not disrupted by cholesterol depletion. They also detected acylated, but not prenylated fluorescent proteins in detergent-insoluble fractions. Taken together, the results of these two studies do indicate that acylated proteins are more likely to be targeted to lipid rafts and caveolae; however, the prenyl chain used by Zacharias *et al.* [64] is not truly representative

Table 1. Lipids and Proteins Targeted to Lipid Rafts and Caveolae

Lipid Rafts	Caveolae	Ref.
Lipid components: Cholesterol Sphingomyelin Glyco-sphingolipid Phosphatidylinositol 4,5-bisphosphate Ganglioside GM1 Ganglioside GM3	**Lipid components:** Cholesterol Sphingomyelin Glyco-sphingolipid Phosphatidylinositol 4,5-bisphosphate Ganglioside GM1	[90, 115, 209, 210, 211, 212]
Structural proteins: Flotillin-1 and -2	**Structural proteins:** Flotillin-1 and –2 Caveolin-1, -2 and –3	[9, 10, 11, 24, 213]
Signalling proteins: H-Ras G_i, G_o, G_β Src kinases Syk kinase Grb2, Erk2 Shc	**Signalling proteins:** H-Ras G_q Src kinases eNOS Phosphatidylinositol 3-kinase Phospholipase C	[32, 42, 48, 49, 56, 57, 115, 160, 214]
Receptors: PDGF receptor EGF receptor IGF-1 receptor TrkA, TrkB	**Receptors:** PDGF receptor EGF receptor IGF-1 receptor TrkA CD36	[56, 96, 115, 128, 136, 137, 215]
GPI-Anchored proteins: e.g. CD59	uPAR	[216, 217]

EGF: epidermal growth factor; eNOS: endothelial nitric oxide synthase; Erk: extracellular signal-regulated kinase; IGF: insulin-like growth factor; PDGF: platelet-derived growth factor; Trk: tyrosine kinase receptor; uPAR: urokinase-type plasminogen activator receptor.

of those found in the cell [65], and so the exclusion of prenylated proteins is not conclusive.

ROLE OF LIPID RAFTS AND CAVEOLAE IN SIGNALLING BY GROWTH FACTOR RECEPTORS

The role of lipid rafts and caveolae in signalling by several growth factor receptors has been studied, but none more so than the epidermal growth factor receptor (EGFR). We will therefore be paying particular attention to this receptor. However, we will also discuss several other receptors for which information is sparser.

1) Epidermal Growth Factor Receptor (EGFR)

Structure and Function of the EGFR

The epidermal growth factor receptor (EGFR) signaling pathway is an important mediator of cancer cell oncogenesis, proliferation, maintenance, and survival [66]. This receptor is expressed in all epidermal and stromal cells as well as some glial and smooth muscle cells, and has several ligands, including EGF itself, transforming growth factor (TGF)-α, and heparin-binding EGF (HB-EGF) [66]. It can also be transactivated by the binding of ligands to certain G-protein coupled receptors, such as the angiotensin II receptor [67]. The EGFR has three known homologues: ErbB2 (Neu or HER2), ErbB3 (HER3) and ErbB4 (HER4), with which it can form heterodimers [68]. Ligand binding causes homo- or heterodimerization of the receptor and activates its tyrosine kinase activity, allowing it both to autophosphorylate and to phosphorylate downstream signalling molecules [69]. The EGFR consists of a number of extracellular domains forming the ligand-binding site [68]. There is then a transmembrane sequence and juxtamembrane domain, followed by an intracellular kinase domain and C-terminal domain. Five autophosphorylation motifs are found in the C-terminal domain, providing docking sites for proteins containing SH2 (Src homology region 2) and phospho-tyrosine binding (PTB) domains (e.g., Grb2). The C-terminus can also act as an autoinhibitory domain when not phosphorylated [66]. Nine phosphorylation sites were described in the EGFR, which differentially regulate the activation of downstream signalling pathways [70, 71]. Firstly there is the well-characterised p42/44 mitogen-activated protein kinase (MAPK) pathway. This begins with the recruitment of Grb2, which contains an SH2 domain and can bind either directly to the phosphorylated EGFR, or to Shc, an adapter protein that becomes associated with and tyrosine phosphorylated by the EGFR. Grb2 is associated with SOS, a Ras guanine nucleotide exchange factor, which is then able to activate membrane-associated Ras [68]. Ras is activated by the exchange of its associated GDP with GTP, and in turn activates Raf-1, a serine/threonine kinase, which leads to the activation of the MAPK pathway. This involves the phosphorylation of MEK (MAP kinase kinase) and the activation and translocation of Erk1 and Erk2 to the nucleus, where they phosphorylate transcription factors such as Elk, stimulating cell proliferation and motility.

Other targets of the EGFR include phospholipase Cγ (PLCγ), phospholipase D (PLD)-1 and –2, and phosphatidylinositol-3-kinase (PI3K). PLCγ hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce diacylglycerol and inositol 1,3,5,-trisphosphate (IP_3), the latter of which releases calcium from intracellular stores. This results in the activation of many calcium-dependent enzymes and downstream pathways [68]. PLCγ can also mobilise actin modifying proteins, enhancing cell motility [66]. PLD-1 may be activated *via* protein kinase Cα [72], while PLD-2 can be activated directly by the EGFR [68]. PLD hydrolyses phosphatidylcholine to produce choline and phosphatidic acid (PA) (Fig. **2**). PA in turn activates mTOR (mammalian target of rapamycin), with which it directly interacts [73]. Among other things, mTOR activates the ribosomal protein S6 kinase 1 (S6K1), which phosphorylates the ribosomal S6 protein, leading to increased cell growth (Fig. **2**). PI3K phosphorylates PIP_2 to produce phosphatidylinositol-3,4,5,-trisphosphate (PIP_3), which can be converted to phosphatidylinositol-3,4-bisphosphate ($PI(3,4)P_2$) by SH2-containing inositol-5-phosphatase (SHIP) [74, 75] (Fig. **2**). PIP_3 and $PI(3,4)P_2$ stimulate the activity the serine/threonine kinase Akt (also known as protein kinase B) by binding to its Pleckstrin Homology (PH) domain. Also required for activation of Akt is its phosphorylation on threonine 308, which occurs *via* phosphoinositide-dependent kinase-1 (PDK1), an enzyme activated by PIP_3 (Fig. **2**). PDK1 can also activate S6K [76]. Phosphorylation on serine 473 is controlled by the rictor-mTOR complex [77]. It is of interest to note here that a lipid raft-associated Akt Ser473 kinase was described [78], and subsequently shown to be DNA-dependent protein kinase (DNA-PK) [79]. This enzyme may also be involved in the regulation of Akt under specific conditions. Akt can phosphorylate proteins such as CREB (cAMP response element binding protein), pro-caspase-9, BAD and the forkhead family of transcription factors (FKHR), and is involved in the regulation of apoptosis, gene expression and cell proliferation [74].

Finally, the activation of Src kinases is associated with activation of the EGFR. Src kinases have numerous substrates, including PI3K and elements of the cytoskeleton [68].

Role of Lipid Rafts and Caveolae in Signalling by the EGFR

The involvement of lipid rafts and caveolae in signalling by the EGF receptor (EGFR) is by no means clear cut. Indeed, there is a great deal of discrepancy in the literature, due in the most part to the use by some researchers of methods which do not allow exclusive separation of lipid rafts and caveolae.

Evidence for EGFR Signalling within Caveolae

Mineo *et al.* [80] used a detergent-free fractionation method to isolate low buoyant density, caveolin-rich membrane fractions from Rat-1 cells, some of which had been treated with EGF. H-Ras, Grb2 and SOS-1 were concentrated in the caveolin-rich fraction, both in unstimulated cells and in those that had been exposed to EGF. On the other hand, they found that Raf-1 was only present in the caveolin fraction following stimulation by EGF, but that this Raf-1 was active. Conversely, the EGFR was present in the caveolin fraction in unstimulated cells, but its levels began to decline after 30 seconds' stimulation by EGF, and following 60 minutes' stimulation it was no longer detectable in this fraction. These results were taken to indicate that EGFRs were present in caveolae, causing them to cluster and therefore allowing efficient dimerization. However, following activa-

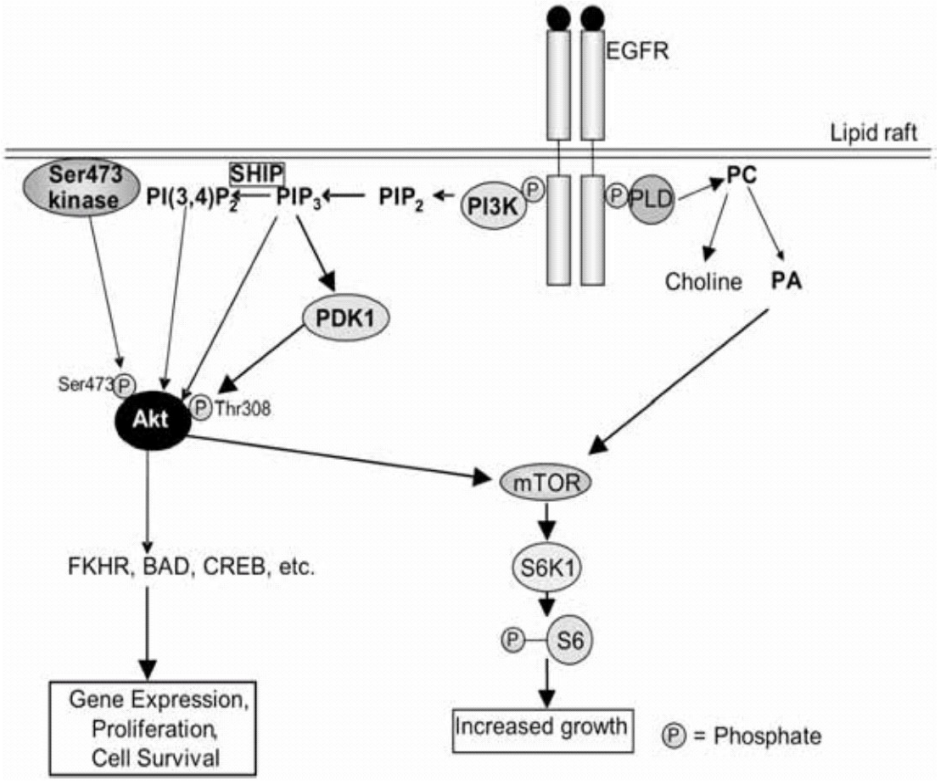

Fig. (2). The EGFR signalling pathway in lipid rafts. See text for description of the pathway. The PLD shown here is PLD-2, which is activated directly by the EGFR. It must be also emphasised that at least three steps contribute to activation of Akt: (i) binding of PIP_3/$PI(3,4)P_2$ to its Pleckstrin Homology domain; (ii) phosphorylation on Thr308 by PDK1; and (iii) phosphorylation on Ser473. PLD: phospholipase D; PA: phosphatidic acid; PC: phosphatidylcholine; mTOR: mammalian target of rapamycin; EGFR: epidermal growth factor receptor; FKHR: Forkhead Box, subgroup O, transcription factors; CREB: cAMP response element binding protein; PDK1: 3-phosphoinositide-dependent kinase-1; PI3K: phosphatidylinositol 3-kinase; PIP_2: phosphatidylinositol-4,5-bisphosphate; $PI(3,4)P_2$: phosphatidylinositol-3,4-bisphosphate; PIP_3: phosphatidylinositol-3,4,5,-trisphosphate; S6K1: ribosomal protein S6 kinase 1.

tion, the EGFR would migrate out of caveolae, allowing termination of the response. Mineo *et al.* [81] obtained similar results for the EGFR, showing that before activation, 60.5% of EGFRs were present in the caveolin fraction of human fibroblasts, but there was a dramatic decrease in number following EGF application.

Jang *et al.* [82] also localised the EGFR in caveolin-rich membrane fractions in unstimulated cells (in this case, A431 and COS-7 cells). Following treatment with EGF, phospholipase Cγ (PLCγ) was recruited to the caveolin fraction. They also used immunostaining of COS-7 cells to visualise the recruitment of PLCγ to the membrane: following EGF stimulation, labelled PLCγ was shown to co-localise with caveolin. In addition, depletion of cellular cholesterol resulted in translocation of the EGFR and caveolin-1 out of the low buoyant density fraction and prevented recruitment of PKCγ to this fraction, as well as inhibiting PIP_2 turnover. However, PLCγ phosphorylation by the EGFR was not decreased upon cholesterol depletion, suggesting that the EGFR can still activate PLCγ when it is delocalised from caveolae. It was therefore hypothesised that loss of PIP_2 turnover was due to its own delocalisation and not that of the EGFR or PLCγ.

A separate study by Han *et al.* [72] placed another downstream effector of EGFR signalling, phospholipase D1 (PLD1) in the caveolin-rich membrane fraction of COS-7 cells. Stimulation by EGF caused phosphorylation of PLD1 in the caveolin-rich fraction, with kinetics similar to which protein kinase Cα appeared in this fraction. Palmitoylation of PLD1 was required for its location within the caveolin-rich fraction, and for its activation *via* the EGFR, which resulted in the local production of the secondary messenger PA.

Evidence that Caveolae Inhibit EGFR Signalling

Several lines of evidence dispute the idea that caveolae are the locations of EGFR signalling, and suggest that caveolae may in fact have a negative regulatory role. More precisely it appears that the scaffolding domain of caveolin-1 stabilizes the EGFR kinase in an inactive conformation [83], and the same negative regulation also exists in the case of the ErbB2 tyrosine kinase activity [84, 85]. Couet *et al.* [83] demonstrated the co-fractionation of the EGFR, and its close relative ErbB2, with caveolin. They went on to show that the EGFR coimmunoprecipitated with caveolin-1, and that the scaffolding domain of caveolin-1 (residues 82-101) could bind to the EGFR *in vitro*. A caveolin-binding motif was identified in the cytoplasmic kinase domain of EGFR, and is conserved in most receptor tyrosine kinases. In addition, the caveolin-1 scaffolding domain dose-dependently inhibited

the kinase activity of the EGFR *in vitro*. The interaction was independent of EGFR activity and did not require tyrosine phosphorylation of caveolin-1.

Interestingly, it has been shown that overexpression of ganglioside GM3 causes increased interaction of the EGFR with caveolin-1 and hence inhibits EGFR signalling [40]. Furthermore, Engelman *et al*. [86] showed that overexpression of caveolin-1 in a fibroblast cell line inhibited signal transduction by the EGFR. Caveolin-1 expression also inhibited the activity of Raf-1, MEK-1 and Erk2, and the scaffolding domains of caveolin-1 and caveolin-3, but not that of caveolin-2, could directly inhibit the activity of MEK-1 and Erk2 *in vitro*. In addition, downregulation of caveolin-1 causes hyperactivation of the same kinase cascade [87].

Zhang *et al.* [88] also provided evidence that caveolae inhibit EGF signalling. They observed that motile mammary adenocarcinoma cells failed to express normal levels of caveolin-1, in contrast to their non-motile counterparts. Expression of caveolin-1 in motile cells inhibited EGF-dependent lamellipod extension and migration in the motile cell line. It was also shown to prevent activation of the p42/44 MAP kinase cascade, which is possibly one mechanism by which it blocks migration.

Further evidence of an inhibitory role for caveolae came from a study on senescent cells by Park *et al.* [89]. Senescent cells show a decreased response to EGFR signalling, and the phosphorylation of Erk1/2 upon EGFR activation was delayed in senescent fibroblasts. Furthermore, they found that the levels of both caveolin-1 and caveolin-2 were significantly increased in senescent cells compared with young cells, and that old fibroblasts contained over 10 times more caveolae. They also reported that all the major organs in old rats showed increased numbers of caveolae in their cells, and that the increase in caveolae numbers was accompanied by an increase in cellular cholesterol levels. In artificially aged cells, which did not overexpress caveolin, the response to EGF was not reduced, as it would be in normally aged cells. Moreover, overexpression of caveolin-1 in young cells inhibited Erk1/2 activation upon EGF stimulation. This suggests that caveolin plays a role in the age-related decrease in EGFR signalling, though other factors might also be involved.

Evidence for the Involvement of Lipid Rafts in EGFR Signalling

Another line of evidence implicates lipid rafts, and not caveolae, in EGFR signalling. Firstly, Waugh *et al.* [90] showed that, although the EGFR was present in the low buoyant density, caveolin-rich fraction from A431 cells, it could not be co-immunoprecipitated with caveolin, and was soluble in Triton X-100, in contrast to caveolin, which was insoluble. Phosphorylated EGFR also did not co-immunoprecipitate with caveolin-1, arguing against a caveolar location for EGFR signalling.

Roepstorff *et al.* [60] showed that depleting cholesterol from the membrane increased ligand binding to the EGFR. Depletion of cholesterol seemed to have this effect by increasing the number of available receptors in the membrane, although the total number of receptors was not changed [91]. It was also demonstrated that oncogenic EGFRs reside in caveolae even after ligand binding, genereting altered signaling pathways such as the enhanced tyrosine phosphorylation of the caveolar molecules, caveolin-1 and dynamin [81]. Li *et al.* [92] demostrated that the depletion of cholesterol also induces rafts disruption with a consequent Bcl-X_L downregulation and Akt inactivation. It is intriguing that EGF administration after caveolae disruption could not restore Akt activation once rafts were disrupted, but cholesterol administration restored Akt activity also in absence of EGF [92, 93].

Roepstorff *et al.* [60] used immunofluorescence to study the relative locations of caveolin and the EGFR on the plasma membrane, and did not find any significant co-localisation. Finally, it was found that although caveolin-1 was insoluble in Triton X-100, the EGFR was soluble in this detergent, but insoluble in another detergent, Brij 58, supporting the idea that it is present in Brij 58-insoluble rafts. It is unknown whether these are a subset of rafts or whether this reflects a certain type of association of the EGFR with rafts [92]. The authors of this paper suggested that sequestration in rafts inhibits ligand-binding to the growth factor receptor, and that depletion of cholesterol allows release of the EGFR from rafts, thereby increasing its activation.

Ringerike *et al.* [94] used immuno-electron microscopy to show that only 7% of EGFRs in A431 cells were localised in caveolae. In contrast to the results reported in [80], they did not find any difference in the distribution of EGFRs following stimulation with EGF. As in Roepstorff *et al*. [60], a co-localisation of the EGFR with markers of lipid rafts was found, and approximately 40% of receptors were present within rafts, which did not change following stimulation with EGF. Depletion of cholesterol increased dimerization of the EGFR, but, in contrast to [60], an increase in EGFR levels at the plasma membrane was observed following cholesterol depletion. Further support came from Ushio-Fukai *et al*. [95], who found that depletion of cholesterol enhanced EGF-mediated autophosphorylation of the EGFR.

Matveev and Smart [96] showed that, although present in the caveolin-rich fraction, only 3% of EGFRs in Swiss 3T3 cells co-immunoprecipitated with caveolin in unstimulated cells, with similar results obtained for cells exposed to EGF for ten minutes. However, the EGFR did co-immunoprecipitate with CD55, a marker of lipid rafts, and was of a molecular weight consistent with tyrosine phosphorylation. Interestingly, in cells stimulated with EGF for 60 minutes, 87-94% of EGFRs co-precipitated with caveolin. At this time, the EGFRs were no longer phosphorylated, which they had been after ten minutes' treatment with EGF. This suggests that signalling might actually occur in rafts, and that sequestration in caveolae following prolonged exposure to ligand may facilitate receptor desensitisation. This is in agreement with the idea that caveolae inhibit EGFR signalling; however, it contradicts the results of Roepstorff *et al*. [60] and Ringerike *et al.* [94], which suggest that rafts also are inhibitory, and is also in direct contradiction to the results of Mineo *et al*. [80, 81].

The results of Zhuang *et al*. [97] suggested an activatory role for lipid rafts in EGFR signalling. This study used the prostate cancer cell line LNCaP, which was shown to contain Triton X-100-insoluble fractions, but not caveolin-1. It was found that, in unstimulated cells, the EGFR was present in detergent-soluble, but not insoluble membranes. Following stimulation with EGF, phosphorylated EGFR appeared in the

detergent-insoluble fraction, but was not detected in the soluble fraction. In addition, treatment with filipin decreased the amount of phosphorylated EGFR in the insoluble fraction following EGF stimulation, but this was restored to normal following the addition of cholesterol. The presence of caveolin also did not alter the levels of Akt activation before cholesterol depletion, suggesting that caveolin-1 might not inhibit this pathway as it does MAPK.

Babuke *et al.* [37] also recently demonstrated a role for reggie-1 in EGFR signaling. After EGFR activation, reggie-1 colocalized with the receptor in the plasma membrane and the endosomes. It was also evident that its Tyr phosphorylation involved Src kinases [98]. A role for reggie-2 in raft-mediated endocytosis has also previously been suggested [99]. These results open the interesting possibility that reggie can also play a role in the raft-mediated endocytosis and signaling of the EGFR [100] which also involves Src kinases [101].

In summary, most evidence indicates an inhibitory role for caveolae in EGFR signalling, which might contribute to the desensitisation of receptors [96]. Furthermore, as Park *et al.* [89] demonstrated that older cells, which have a reduced response to EGF, have elevated numbers of caveolae, it is likely that one way in which cells can control the level of signalling by the EGFR is by regulating their number of caveolae. However, it is possible that caveolae only inhibit certain downstream pathways, such as MAPK, and not others, such as PI3K/Akt. There is a certain amount of disagreement in the data concerning whether lipid rafts play an inhibitory or activatory role in EGFR signalling, in that some researchers reported an increased level of signalling following cholesterol depletion, while others have located activated EGFRs in lipid raft fractions. Given that the cells used by Roepstorff *et al.* [60] and Ringerike *et al.* [94] also contained caveolae, however, and the fact that phosphorylated EGFRs were clearly localised to rafts by Zhuang *et al.* [97] and Matveev and Smart [96], it is likely that lipid rafts could play an activatory role in EGFR signalling. The bulk of the evidence suggests that the EGFR is constitutively present in rafts, but that its association with rafts is probably strengthened following activation, causing it to become insoluble in Triton X-100. It could then initiate signalling pathways in these domains, which contain a number of molecules downstream from the receptor.

Association of Downstream Signalling Pathways with Rafts

Assuming that EGF signalling is localised to rafts, it is necessary to note that not all the downstream molecules may act in these domains. For example, there is evidence to suggest that activated Ras might act outside of rafts. There are three isoforms of Ras: N-Ras, H-Ras and K-Ras, of which K-Ras is most efficient at activating Raf, while H-Ras efficiently activates PI3K [102]. Fukano *et al.* [103] used immunogold labelling to show that some H-Ras, but not K-Ras, was localised to caveolae, and that non-caveolar H-Ras colocalised with a marker of lipid rafts, while K-Ras did not. However, activated H-Ras (G12V) was almost completely excluded from the caveolae/raft fraction. More recently, Prior *et al.* [104] reported that H-Ras exists in a dynamic equilibrium between lipid rafts and non-raft membrane. However, constitutively active H-Ras (G12V) was not localised to lipid rafts, but to cholesterol-independent microdomains. They also examined the distribution of K-Ras, and found that it was localised to cholesterol-independent microdomains. Together, these results indicate that K-Ras and H-Ras occupy different microdomains, and that activated Ras is not associated with rafts.

Given that activated Ras is excluded from rafts, presumably it activates Raf outside of rafts, and it seems strange that Mineo *et al.* [80] found phosphorylated Raf-1 in rafts, suggesting that it returns to these domains upon activation. However, Chen and Resh [105] created several Raf-1 constructs that localised to rafts to different extents, and the extent of localisation to rafts did not affect the extent of Erk activation, Erk being excluded from rafts. The cholesterol depletion data gave varying results: Peiro *et al.* [106] found that cholesterol depletion did not affect MAPK activation by the EGFR, while Chen and Resh [105] found that MAPK activation was increased, but that this occurred *via* PI3K. They suggest that it is due to factors other than raft disruption. Furuchi and Anderson [107] also detected increased EGF-stimulated Erk activation following cholesterol depletion. These results are complicated by the presence of caveolae, and the recent discorvery of a cholesterol-regulated Erk phosphatase [108]. Therefore the evidence suggests that, although H-Ras is probably activated in rafts initially (given that the EGFR, Grb2, Sos and Shc have been found in rafts [57, 80]), activated Ras moves out of rafts to activate Raf, and a raft location for Raf is not strictly required for activation of Erk. However, as mentioned in below sections, studies of other systems have shown that cholesterol depletion can inhibit the MAPK pathway, suggesting that this may vary depending on the receptor involved.

Alternative pathways are related to Ras activation and can act in balance with the EGFR, one of the most important already described is the presence in lipid rafts of the Spred-1 protein. This protein, recruited to the lipid raft/caveolae, efficiently interacts with Ras and also Raf-1 by interacting *via* caveolin-1, resulting in a strong inhibition of the Ras/ERK pathway [109].

As mentioned above, the activation of PLCγ by EGF signalling is not affected by cholesterol depletion, suggesting that a raft location is also not required for the activation of this pathway [82]. However, this study also showed that PIP_2 turnover was dependent on rafts. There is also evidence that PLD is activated in rafts: Han *et al.* [72] localised activated PLD in caveolae/raft fractions following stimulation with EGF. In addition, there is evidence that the PI3K/Akt pathway is localised to rafts, as cholesterol depletion prevented EGF-induced phosphorylation of Akt [92]. Furthermore, PIP_2, a substrate of PI3K, has been found in rafts [110], as has the Akt Ser473 kinase [78]. Hill *et al.* [78] therefore hypothesise that a constitutively active Ser473 kinase is present in rafts, and the activation of PI3K (e.g., by the EGFR) and subsequent generation of PIP_3 from PIP_2 results in the recruitment of PDK1 and Akt to rafts.

In summary, most of the published evidence suggests a raft localisation for PLD and the PI3K pathway. In particular, as cholesterol depletion prevents Akt phosphorylation [92], a raft localization may be required for adequate functioning of this pathway. In contrast, Ras acts outside of rafts, and while activated Raf and PLCγ have been found in rafts, they need not be present in rafts in order to signal normally.

Therefore, different pathways downstream from the EGFR might be differentially dependent on lipid rafts for their activation.

2) Platelet-derived Growth Factor Receptor (PDGFR)

Structure and Function of the PDGFR

There are two types of PDGFR: the α-receptor (PDGFRα) and the β-receptor (PDGFRβ), which bind different PDGF types and have distinct but overlapping cellular distributions [111]. The two receptors have a similar structure, possessing five immunoglobulin-like domains extracellularly, and in the intracellular region a juxtamembrane domain, tyrosine kinase domain and C-terminal domain. This is reminiscent of the EGFR; however, a distinguishing feature of the PDGFR is that its kinase domain is split in two by a non-kinase 100 amino acid insert [111, 112]. Upon binding of ligand, the receptor dimerises, and can form either homo- or heterodimers, depending on the isoform of PDGF [112]. As for the EGFR, activation results in autophosphorylation of the receptor, increasing its kinase activity and providing docking sites for proteins with SH2 domains. Downstream molecules activated by the PDGFR include PI3K, PLCγ, several members of the STAT family and the non-receptor tyrosine kinase Src. It can also activate the Ras/MAPK pathway *via* Grb2 and SOS [111]. Activation of the PDGFR provokes several responses: early responses include intracellular Ca^{2+} flux and the reorganisation of the cytoskeleton, resulting in the formation of plasma membrane processes. Later effects include cell migration, proliferation and differentiation [112].

Involvement of Lipid Rafts and Caveolae in Signalling by the PDGFR

Again there are a number of conflicting results concerning the role of lipid rafts and caveolae in PDGFR signalling. Some studies suggest that caveolae are the sites of signalling; some suggest that caveolae are inhibitory and that signalling occurs in rafts.

Liu *et al.* [113] localised the PDGFRβ to the low buoyant density, caveolin-rich fraction of human fibroblasts, indicating that it was present in either caveolae or rafts. They then used immunocytochemistry to demonstrate that the PDGFRβ was found in distinct patches on the cell surface, and that it co-localised with caveolin in caveolae-like structures. Following stimulation with PDGF, phosphorylated PDGFRβ was found in the caveolin-rich fraction, as were increased amounts of the tyrosine phosphatase Syp, Shc, and MAPK. However, it is important to note that this fraction will also have contained lipid rafts.

Liu *et al.* [114] first showed by immunoblotting that the PDGFRβ, Ras, Raf-1, MEK-1 and Erk2 were all present in caveolae/raft fractions from unstimulated human fibroblasts. The localization of Erk2 and the PDGFR in caveolae was confirmed using immunocytochemistry. Stimulation with PDGF resulted in accumulation of a number of phosphorylated proteins, including activated MAPK, in the caveolae/raft fraction. Immunogold labelling seemed to confirm the presence of tyrosine-phosphorylated proteins in caveolar structures following but not prior to PDGF treatment. Liu *et al.* [115] used a technique designed to separate caveolae from lipid rafts, and found the PDGFRβ to be associated with these domains, along with molecules known to act downstream of the receptor, such as PLCγ, PI3K and non-receptor tyrosine kinases. In addition, they used confocal immunofluorescence microscopy to show that PDGF treatment caused tyrosine phosphorylation of proteins in caveolae, both in cell culture and in intact rat lungs. Disruption of caveolae by cholesterol depletion prevented PDGF-induced tyrosine phosphorylation; the PDGF receptor itself was phosphorylated, but less so than in the control. On the other hand, tyrosine phosphorylation by vanadate was not reduced by cholesterol depletion. Again it must be noted that cholesterol depletion also affects rafts, and can often give unreliable and conflicting results.

The results related above suggest a caveolar location for PDGF and at least some PDGF-induced tyrosine phosphorylation. However, this does not agree with the observation of Couet *et al.* [83], since the PDGFR contains a motif associated with binding to the scaffolding domain of caveolin, an interaction which is usually inhibitory. Furthermore, Yamamoto *et al.* [116] showed that both the PDGFRα and PDGFRβ, which were present in the caveolae/raft fraction, could be immunoprecipitated with caveolin-1. Moreover, the phosphorylation of PDGFRαs was inhibited in a dose-dependent manner by the scaffolding domains of both caveolin-1 and caveolin-3, but not caveolin-2.

Work by Matveev and Smart [96] also suggested an inhibitory role for caveolae, and localised PDGF signalling to rafts. They showed that, although the PDGFR was located in the caveolin-rich, caveolae/raft fraction, it coimmunoprecipitated with CD55, a marker of non-caveolar rafts, and only 3% co-immunoprecipitated with caveolin. Following treatment with PDGF, the PDGFR that co-immunoprecipitated with CD55 was of a molecular weight consistent with tyrosine phosphorylation; little PDGFR precipitated with caveolin. However, most of the PDGFR co-immunoprecipitated with caveolin after exposure to PDGF. This is similar to the results obtained for the EGFR in the same paper. Indeed, antibodies to the PDGFR precipitated membranes containing the EGFR and vice versa, suggesting that the two receptors are present in the same microdomains. This is supported by the fact that Matveev and Smart [96] showed that pre-treatment of Swiss 3T3 cells with either EGF or PDGF for 60 minutes caused sequestration of the receptor for the other ligand, preventing ligand binding and activation. Filipin and the cholesterol-depleting agent methyl-β-cyclodextrin allowed re-exposure of the same receptors. Pre-treatment with either of these substances also prevented sequestration of either receptor. Sequestration was also prevented in cells lacking caveolin-1, supporting a role for caveolae. This indicates that cross-talk between the PDGFR and EGFR occurs, supporting the hypothesis that they are present in the same microdomains, and implicates caveolae as sites of sequestration.

The results just reported are very difficult to reconcile. The results of Liu *et al.* [113-115] suggest that PDGF signalling occurs in caveolae; however, the more recent study by Matveev and Smart [96] suggests that the PDGFR is normally located in lipid rafts, and re-localises to caveolae after prolonged exposure to ligand. In support of a role for lipid rafts is the cross-talk that occurs between the PDGFR and

the EGFR, suggesting that they are present in the same microdomains. As discussed above, most evidence suggests that the EGFR is associated with rafts.

It is possible that some PDGFR is present in lipid rafts and some in caveolae. Although Liu *et al.* [113, 114] demonstrated that the PDGFR was present in caveolae, their immunocytochemistry/immunogold labelling do show that it is also present outside of caveolae; as it was detected mainly in caveolae/raft fractions, it is therefore likely to be also present in rafts. In addition, the tyrosine-phosphorylated proteins detected by Liu *et al.* [113, 115] were present in the caveolae/raft fraction and could have been associated with rafts.

In summary, it is not possible to say with certainty what the role of lipid rafts and caveolae is in signalling by the PDGFR, although the location of phosphorylated proteins in the caveola/raft fraction, including MAPK [114] suggests that at least one of these domains is involved in signalling. This is supported by a paper by Mitsuda *et al.* [117], who showed that overexpression of the ganglioside GM1 resulted in the re-location of the PDGFR to non-raft/caveolar membrane and hence an inhibition of PDGFR signalling.

3) c-Kit- the Stem Cell Factor Receptor

c-Kit is a type III receptor protein-tyrosine kinase, in a class which also includes the PDGFR, and the CSF-1R. Its extracellular N-terminal domain binds to stem cell factor (SCF) and then induces the dimerization of the receptor resulting in activation of the cytoplasmic catalytic activity. This cytoplasmic domain is needed, together with adaptor proteins such as Grb2 and Grb7, to activate several downstream pathways. One of the most important downstream mediators of c-Kit is PI3K, which then activates Akt, a protein-serine/threonine kinase, promoting cell survival [118, 119]. Other downstream effectors of c-Kit include the Ras/mitogen-activated protein kinases and activators of transcription (Jak/STAT) pathways [120].

Lipid raft clustering was shown after SCF stimulation of hemoatopoietic stem cells (HSCs) and correlated with activation of the Akt-FOXO signalling pathway [121]. Inhibition of lipid raft clustering attenuated SCF signalling and prevented hibernating HSCs from re-entering the cell cycle [121]. Jahn *et al.* [122] showed the translocation of c-Kit to lipid rafts after activation of the receptor by ligand binding. The receptor was then co-localized with signalling mediators such as PDK1 and Akt that transduce the survival signal of SCF/c-Kit [122]. Arcaro *et al.* [110] presented evidence that SCF stimulation induces Src kinase activation in the detergent-insoluble fraction of small cell lung cancer (SCLC) cell lines. Src kinase activity was required for optimal Akt activation, which was supported by the fact that Src and PI3K associated in the lipid rafts fraction of SCLC cells [110]. They also demonstrated that on the other hand, the activation of the Erk pathway after SCF stimulation is independent from the integrity of lipid rafts [110, 123].

4) Insulin-like Growth Factor-1 Receptor (IGF-1R)

The IGF-1R binds to and mediates the cellular effects of IGF-1, and also binds with lower affinity to IGF-2 [124]. Like other tyrosine kinase receptors, it contains extracellular ligand-binding and cysteine-rich domains, a transmembrane domain and an intracellular kinase domain. However, it is unusual in existing as a dimer on the cell surface prior to stimulation, and so depends on domain rearrangements rather than dimerisation in order to initiate signalling [124]. Downstream pathways activated by the IGF-1R include the p42/44 MAPK pathway and the PI3K/Akt pathway. The IGF-1R is expressed in a wide range of tissues, and its activation can lead to proliferation, differentation and the prevention of apoptosis [124].

Ravid *et al.* [125] demonstrated that constitutive overexpression of caveolin-1 in MCF-7 breast cancer cells inhibits anoikis by attenuating p53 and p21 activation. From this observation they went further, demonstrating that Akt substrate (pp340) phosphorylation is constitutively present, activating the protein and the signalling through it. The signal through the ERK1/2 pathway was also increased [126]. This study together with others demonstrates that IGF-1R stimulation induces anchorage-independent survival of the cells, through caveolin-1 and Akt, leading to a highly motile phenotype of MCF-7 cells [125, 126].

Podar *et al.* [127] found that depletion of cellular cholesterol prevented recruitment of PI3K to caveolae/rafts by IGF-1, inhibited IGF-1-induced IRS1 (insulin receptor substrate 1 – an adapter protein recruited by the IGF-1 receptor) phosphorylation, and blocked IGF-1-stimulated PI3K and Akt activation, but not Erk phosphorylation. However, cholesterol depletion data are complicated by a number of factors, including the existence of a cholesterol-regulated Erk phosphatase [108], and so are not too reliable. Nevertheless, the fact that Erk was not found in the caveolae/raft fraction indicates that it does not signal in these domains, in support of results reported above.

Maggi *et al.* [128] overexpressed human IGF-1R in mouse fibroblasts, and localised the IGF-1R to the caveolin-rich fraction upon ultracentrifugation in a sucrose gradient. They also showed that IGF-1 stimulation resulted in phosphorylation of caveolin-1 on tyrosine 14. Furthermore, IGF-1 seemed to cause translocation of caveolin-1 from the fraction in which it is normally present (fraction 7) to the lighter fractions 5 and 6, which are thought to contain lipid rafts.

Huo *et al.* [129, 130] also found that IGF-1 was present in the caveolae/raft fraction of 3T3 preadipocytes, and showed by double immunofluorescence staining that the IGF-1R co-localised with caveolin-1α in these cells. Furthermore, the IGF-1R was shown to co-immunoprecipitate and to interact with caveolin-1, and caveolin associated with the activated IGF-1R just as it did with the quiescent receptor. This seems to suggest that caveolin-1 does not, in this case, prevent receptor activation. Because the IGF-1R was present not only in fractions containing caveolin but also in some lighter fractions, Huo *et al.* [129, 130] suggested that it was also present in non-caveolar lipid rafts. It was shown that disrupting lipid rafts and caveolae by cholesterol depletion inhibited IGF-1-induced differentiation of preadipocytes into adipocytes, as well as IGF-1-induced clonal expansion. This was not due to inhibition of receptor activation, but to an inhibition of the downstream molecules Erk1 and Erk2. These results are however in contrast with those of Matthews *et al.* [131], who concluded that rafts were not involved in activation of Erk1/2, and that in general they are not essen-

tial components in the transduction of the biological actions of IGF-1.

The results of Huo *et al*. [129], who found that lipid rafts/caveolae are required for IGF-1-induced adipocyte differentiation, are interesting in light of the results of Razani *et al*. [132]. These researchers fed caveolin-1 knockout mice on a high fat diet, and found that these mice were resistant to diet-induced obesity, and showed reduced adiposity as they grew older. As Huo *et al*. [129] point out, this suggests that these mice have abnormal adipocyte differentiation in adulthood, and supports a role for caveolae in adipocyte differentiation, possibly *via* the IGF-1R.

In summary, the data so far suggests that the IGF-1R is associated possibly with both lipid rafts and caveolae, and that one or both of these domains are involved at least in downstream signalling from the receptor. Further work is clearly required to determine whether rafts and caveolae have a distinct role in IGF-1R signalling, and for example to determine the significance of the observed translocation of caveolin-1 into supposed lipid raft fractions following IGF-1 stimulation [128].

5) Nerve Growth Factor (NGF) Receptors (TrkA and $p75^{NTR}$)

NGF is a member of the neurotrophin family of growth factors, and can activate two receptors: TrkA and the p75 neurotrophin receptor ($p75^{NTR}$). TrkA is a tyrosine kinase receptor, through which NGF signals cell survival, and $p75^{NTR}$ is a member of the tumour necrosis factor receptor family, which induces cell apoptosis [133]. TrkA has a similar structure and function to the tyrosine kinase receptors discussed above; it can associate with the adapter protein Shc, PI3K and PLCγ, and is capable of activating the Ras/MAPK pathway [134]. The activation of $p75^{NTR}$ may result in the generation of ceramide (*via* sphingomyelin hydrolysis), activation of the JNK (Jun N-terminal kinase) pathway and activation of the transcription factor NF-κB [135].

Bilberback *et al*. [136] demonstrated that $p75^{NTR}$ was present in the caveolae/raft fraction from p75-NIH 3T3 cells, and that it co-immunoprecipitated with caveolin. They also localised $p75^{NTR}$-induced sphingomyelin hydrolysis to this fraction. Wu *et al*. [56] localised TrkA to low buoyant density fractions lacking caveolin, suggesting that it is present in rafts, and Huang *et al*. [137] demonstrated that 40% of TrkA and 60% of $p75^{NTR}$ in 3T3-TrkA-p75 cells was present in the caveolae/raft fraction. $p75^{NTR}$ co-immunoprecipitated with caveolin either with or without NGF treatment; however, the co-immunoprecipitation of TrkA with caveolin could not be demonstrated. Possibly the interaction was of low affinity, as the results of Bilberback *et al*. [136] (see below) suggest that TrkA can interact with caveolin. Cholesterol depletion using filipin altered the distribution of TrkA and inhibited its phosphorylation by NGF.

Huang *et al*. [137] also used PC12 cells, and could not detect caveolin-1 in these cells; however, in the paper dicussed below, Peiro *et al*. [106] did detect caveolin and caveolae in PC12 cells. This issue is therefore controversial. Nevertheless, Huang *et al*. [137] showed that TrkA and $p75^{NTR}$ were present in low buoyant density (caveolae/raft) fractions in these cells in similar proportions to those in 3T3-TrkA-$p75^{NTR}$ cells. Following treatment with NGF, NGF-bound TrkA and $p75^{NTR}$ were detected both in the low buoyant density fractions and other membrane fractions. However, more NGF-bound receptor seemed to be present in the low buoyant density fractions, and these fractions seemed to be enriched in higher affinity receptors, especially with regard to TrkA. Furthermore, activated TrkA was enriched in these fractions compared to other fractions following NGF stimulation. Huang *et al*. [137] then examined downstream molecules of TrkA, and found that, although not present solely in the caveolae/raft fraction, the adapter molecule Shc only co-immunoprecipitated with activated TrkA in this fraction. A similar result was found for PLCγ. This suggests that NGF signalling *via* these molecules at least is localised to caveolae or rafts.

Peiro *et al*. [106] used double immunofluorescence microscopy and immunogold techniques to show that a fraction of TrkA co-localised with caveolin and was present in the caveolae of PC12 cells. Depletion of cholesterol resulted in an increased basal level of MAPK activity, but inhibited the activation of MAPK by TrkA, although not affecting NGF-induced phosphorylation of TrkA. This also suggests an involvement of caveolae and/or lipid rafts in TrkA signalling. In addition, it suggests that the requirement for lipid rafts for MAPK activation may depend on the system studied and receptor involved, as in the same study it was found that EGF-stimulated MAPK activation was not affected by cholesterol depletion. As emphasised above, however, other factors may be involved.

On the other hand, the results of Bilberback *et al*. [136] suggest an inhibitory role for caveolae. They showed that transfection of PC12 cells (which they found to express only very low endogenous levels of caveolin-1) with caveolin-1 suppressed NGF-induced differentiation, which usually results in the development of neurites. In addition, although TrkA was autophosphorylated both in these cells and normal PC12 cells after 15 minutes' treatment with NGF, after this time TrkA autophosphorylation was rapidly abrogated in cells that had been transfected with caveolin. They also demonstrated that TrkA interacted with caveolin-1, as did $p75^{NTR}$, and an *in vitro* kinase assay indicated that caveolin-1 inhibited NGF-induced TrkA autophosphorylation. The authors conclude that caveolae play a negative regulatory role in TrkA signal transduction.

More recently, additional studies about Trk receptor family (TrkA, TrkB and TrkC) were published, especially regarding the neuronal signaling cascade where these receptors are essential [56, 138]. Limpert *et al*. [139] demonstrated that following NGF stimulation, TrkA is concentrated whithin the lipid raft fraction of the plasma membrane. This is possibly due to the adapter function of CAP, which links TrkA-containing complexes to flottilin. It was also recently demonstrated that there is an interaction of TrkA and TrkB with Src family kinases, such as Fyn and Src [140, 141].

The fact that Bilberback *et al*. [136] found that caveolin inhibits TrkA signalling suggests that rafts might be the more likely location of signalling, similar to the situation with the EGFR. Less information is available for $p75^{NTR}$, but $p75^{NTR}$-induced sphingomyelin hydrolysis at least has been localised to either rafts or caveolae. The results above sug-

gest anyway, that TrkA, and possibly $p75^{NTR}$, are present in both rafts and caveolae. They also suggest that activation of Shc, PLCγ and MAPK by TrkA occurs in either lipid rafts of caveolae.

6) Fibroblast Growth Factor Receptor (FGFR)

There are now four known FGFRs, designated FGFR1-4, all of which are tyrosine kinase receptors. FGFR activation can lead to activation of PLCγ, Src kinases and FGF receptor substrate 2 (FRS2). FRS2 in turn recruits Grb2, leading to the activation of the Ras/MAPK pathway, and can also activate protein kinase C [142]. FGF signalling has a number of effects, including the promotion of angiogenesis, wound healing and limb development. Unfortunately, only two studies have been performed concerning the role of lipid rafts and caveolae, as we will summarise below.

Davy *et al*. [143] showed that FGF-2 was able to induce the tyrosine phosphorylation of proteins in the caveola/raft fraction of human neuroblastoma (LAN-1) cells. Some of this phosphorylation required Src kinases, of which Lyn and Fyn were found in the caveolae/raft fraction, and were phosphorylated following FGF-2 application. In addition, a downstream substrate of Src kinases, annexin II, was recruited to the raft fraction upon FGF-2 treatment. The authors noted that FGFR-2 was present in these cells, but that it was soluble in Triton X-100; however, it does not necessarily follow that FGFR-2 was not present in rafts/caveolae. It has been shown, for example for the EGFR [60], that proteins associated with rafts may be differentially soluble in different detergents.

Ridyard and Robbins [138] showed that FGF receptor substrate 2 (FRS2) was present in caveolae/rafts from LAN-1 cells. FRS2 became serine-threonine phosphorylated following FGF-2 stimulation, and this required the activation of Src kinases, PKC, and MEK-1/2, suggesting an involvement of the MAPK cascade. FRS2 was also tyrosine-phosphorylated following FGF-2 treatment. The authors also observed that stimulation with FGF-2 resulted in the recruitment of Grb2, which is downstream of FRS2, to the raft fraction. Enhancing the tyrosine phosphorylation of FRS2 increased the recruitment of Grb2. The fact that FRS2 is myristoylated [144] may contribute to its localisation to rafts.

Both of the above studies suggest that membrane microdomains are involved in FGF signalling and activation of Grb2 and FRS2.

CONCLUSIONS

Unfortunately, for many of the growth factor receptors discussed, little information is available concerning the role of lipid rafts and caveolae in signalling, and, in addition, much of the data exist is contradictory. This is due in part to the use of methods which do not completely isolate lipid rafts and caveolae, and the use of methods such as cholesterol depletion, which can give unreliable results. Nevertheless, evidence does exist for the involvement of either lipid rafts or caveolae in some aspect of signalling for all the receptors discussed. For caveolae this role seems often, though possibly not always, to be an inhibitory one, due to the inhibitory interactions of caveolae with various proteins, including the EGFR, TrkA, and components of the MAPK pathway. On the other hand, caveolae may not be capable of inhibiting some other downstream pathways, such as PI3K/Akt.

Many studies suggest that membrane microdomains can also act as 'signalling platforms' for growth factor receptors. For example, activated growth factor receptors and downstream molecules have been located in caveolae/raft fractions, and in some cases cholesterol depletion has been shown to inhibit growth factor signalling (e.g., see PDGF, IGF-1, NGF). The fact that caveolin-1 inhibits several growth factor receptors and their downstream molecules makes it more likely that rafts in general are the sites of signal initiation. However, it is possible that caveolae may have a role in downstream signalling from the PDGFR, and caveolin has not been shown to inhibit the IGF-1R. An added complication is the fact that only some downstream molecules require rafts to signal: these include the PI3K/Akt and PLD pathways. With regard to the MAPK pathway, the situation seems to be more complicated: activated Ras seems to move out of rafts, but there is disagreement as to whether Erk activation requires rafts. Possibly this depends on the system studied, but the results are complicated by the many effects of cholesterol depletion.

Clearly much further work is required to determine the exact role of rafts and caveolae in signalling from the growth factor receptors mentioned. The EGFR is the only receptor for which quite a large amount of information is available, and the bulk of the evidence suggests that caveolae inhibit signalling, while lipid rafts are likely to act as signalling platforms at least for signalling *via* PLD1 and PI3K/Akt.

INVOLVEMENT OF LIPID RAFTS AND CAVEOLAE IN MALIGNANT TRANSFORMATION

The fact that lipid rafts and caveolae have been implicated in signalling by growth factor receptors, which are involved in the regulation of proliferation, differentiation, apoptosis and cell migration, suggests the possibility that the alteration of these domains could be involved in malignant transformation. So far, most of the evidence for this theory relates to caveolae, and, as we will describe, caveolin-1 has been identified as a possible tumour suppressor gene. Firstly, however, we will discuss a possible role for lipid rafts in cancer.

1) Lipid Rafts

The central role that lipid rafts play in cancer development and metastasis has become evident only in the recent few years. Lipid rafts are areas where many growth factor receptors have been shown to localize, and thus it was obvious to think of these domains as a possible location where cell signalling events can be altered. Some reports such as Liu *et al*. [145] have provided clear evidence that disruption of rafts inhibits EGF-induced chemotaxis of human breast cancer cells. Further investigations also revealed impared directional migration of cells, EGF-induced cell adhesion, actin polymerization and also translocation of some receptors.

Upregulation of the PI3K pathway in cancer often occurs due to a defect in the *PTEN* (phosphatase and tensin homologue deleted on chromosome ten) tumour suppressor gene,

which is mutated in numerous cancers [146]. PTEN dephosphorylates phosphatidylinositol phosphates (e.g., PIP_3, $PI(3,4)P_2$), and when this activity is lost it can lead to constitutive activation of Akt, and increased cancer cell survival and proliferation. Moreover, the *PIK3CA* gene, encoding the catalytic p110α isoform of PI3K is mutated in a variety of human cancers [147, 148]. These mutations constitutively activate the catalytic activity of PI3K and are oncogenic [149-151]. Consistent with this, Zhuang *et al.* [97] reported that an inhibitor of PI3K induced apoptosis of LNCaP cells, while the effect of this drug was reversed by treatment with EGF. They localised the activated EGFR to rafts, and treatment with filipin induced apoptosis to a similar extent as the PI3K inhibitor, and under these conditions EGF pretreatment could not prevent apoptosis. It was shown that filipin prevented constitutive and EGF-stimulated phosphorylation of Akt1, explaining its ability to induce apoptosis and prevent EGF-mediated survival. The authors interpret these results to mean that Akt signalling in these cells is still dependent on upstream signalling from the EGFR, and that EGFR signalling through Akt is dependent upon cholesterol-rich lipid rafts. Transfecting cells with caveolin-1 did not change the effects of cholesterol depletion on EGFR/Akt signalling, suggesting that caveolae are not involved, and furthermore that lipid rafts have a similar effect in cells that do possess caveolae.

The results of Zhuang *et al.* [97] are interesting in the light of the fact that a link has been found between high fat intake and risk of prostate cancer [152-154], and Zhuang *et al.* [97] suggest that this might be related to high cholesterol levels. While depleting cholesterol disrupts rafts and decreases EGFR signalling, increasing cholesterol levels increases the size of rafts [155] and possibly raft number.

Other receptors that are known to accumulate in lipid rafts are involved in the process of malignant transformation. Recently several authors have also focalized on the IGF-1R [156-158]. In the case of this receptor, most of the the evidence seems to go into the direction of alterations in the PI3K/Akt pathway. Constitutively active TrkA mutants can also cause cellular transformation (e.g., in colon carcinoma or acute myeloid leukaemia) [159], and the PDGFR has been observed to be up-regulated or mutated in cancer [111]. Rafts also contain a number of other proteins that can cause cancer if mutated or misregulated, including PI3K, Ras, and Src. While it has been shown that constitutively active H-Ras is excluded from rafts [160], rafts could act to stabilise signalling from other oncogenic proteins like those just mentioned. Furthermore, if cholesterol levels were increased in cells transformed by these proteins, this could stimulate their signalling still further, exacerbating the problem. The recent finding that lipid rafts are the place where all these altered growth factor receptors initiate their signals, suggests that lipid rafts could be the target for new drug development.

Therefore Zhuang *et al.* [97] suggest that cholesterol depletion could be a useful treatment for prostate cancer, and refer to a study by Gordon and Schaffner [161], who found that sterol-binding agents such as filipin reduced prostate gland hyperplasia in dogs with no toxicity. In support of the use of cholesterol depletion for cancer treatment are the results of Podar *et al.* [127]. Cholesterol depletion inhibited IGF-1-induced activation of PI3K and Akt in multiple myeloma cells, and induced G_1 growth arrest. Given that mutation in *PTEN* and *PIKCA* are commonly observed in human cancer [146, 147], the role of lipid rafts in activation of the PI3K/Akt pathway may be important in numerous malignancies. However, there is still the possibility of cholesterol depletion agents causing toxicity in humans, and *in vivo* studies of tumours are required to confirm the work of Zhuang *et al.* [97] regarding the link between high cholesterol, elevated signalling through the EGFR, and prostate cancer incidence/progression. Intriguingly, caveolin-1 is often up-regulated in prostate cancer cells [162], and its expression is known to be increased by high levels of cholesterol [22]. High cholesterol could therefore be the reason for this. However, as explained below, in other cell types caveolin-1 can act as a tumour suppressor, and cholesterol depletion has been shown to decrease caveolin-1 levels [23], so this treatment might have deleterious effects in these types of cancer and would have to be used specifically.

Several already known anti-cancer agents have recently been found to inhibit tumor cells proliferation, or spreading through the disruption of lipid rafts components [163]. For instance, Huang *et al.* [163] have recently discoverd that Emodi, the major active component of *Rheum palmotum* L., with known anti-cancer activities, is able to induce a significant decrease in cholesterol and sphingolipids in the raft fraction. Data from their study proved that Emodin, through the impairement of lipid raft-associated integrin signaling pathways inhibits adhesion and spreading of tumor cells. Similar results were found using different tumor types [164, 165] and other drugs [166-168].

2) Caveolae

The fact that caveolin-1 is capable of suppressing the activity of many components of growth factor signalling pathways (e.g., the EGFR and components of the p42/44 MAPK pathway [86, 88]), makes it an attractive candidate tumour suppressor gene, and several lines of evidence support this proposal.

Caveolin-1 is Down-Regulated in Tumours and Tumour Cell Lines

Caveolin-1 has been observed to be down-regulated in a number of tumours and tumour cell lines. Firstly, Koleske *et al.* [169] showed that caveolin-1 mRNA was down-regulated in NIH 3T3 fibroblasts that had been transformed by a number of different oncogenes including v-Abl and H-Ras (G12V). Transformation of cells by v-Abl occurs at least partly *via* the Ras/MAPK pathway [170]. The transformed cells were examined under the electron microscope and observed to contain few if any morphologically identifiable caveolae. In addition, Roussel *et al.* [171] found that very low levels of caveolin-1 were present in several lung cancer cells lines, although in most cases caveolin-2 was present at normal levels [172]. Caveolin-2 was shown to be unable to form caveolae in the absence of caveolin-1 [13], and in this case is also not transported out of the Golgi. The real function of caveolin-2 is still unknown but Webley *et al.* [173] suggest the evidence of its role as associated with the inclusions of obligate intracellular pathogens.

Cassoni *et al.* [174] also demonstrated an evident association between tumor progression and a more structured

membranous pattern of caveolin-1 expression. This evidence appears in brain tumor cells derived from 64 patients [174]. Different groups found caveolin-1 to be down-regulated in tumours derived from the ovary, breast and colon [162, 175]. In ovarian carcinoma cell lines this down-regulation required DNA methylation, and transfection of ovarian cancer cells with caveolin-1 decreased cell survival. While two carcinoma cell lines did not down-regulate caveolin-1, they found the caveolin-1 in these cells to be phosphorylated on tyrosine 14. Phosphorylation of caveolin-1 on tyrosine 14 (e.g., by Src) has been shown to stimulate anchorage-independent growth and cell migration [176]. In addition, Wiechen *et al.* [162] found that caveolin-1 was down-regulated in numerous human sarcomas, and interestingly found that treatment with an inhibitor of MEK potently up-regulated the expression of caveolin-1α in a fibrosarcoma cell line, while in this case interfering with methylation had little effect.

Re-Expression of Caveolin-1 Inhibits Anchorage-Independent Growth

Engelman *et al.* [177] expressed caveolin-1 in v-Abl- and H-Ras (G12V)-transformed NIH 3T3 cells (which normally lack caveolin), and found that this abrogated the growth of these cells in soft agar. In v-Abl-transformed cells, caveolin-1 expression also led to visible cell death. They also discovered that expression of caveolin-1 inhibited H-Ras (G12V) and MAPK-dependent activation of the c-fos promoter (as determined by expression of a reporter gene attached to the promoter). Furthermore, transfection of F11 cells with caveolin-1 lead to nucleosomal DNA fragmentation, a hallmark of apoptotic cell death. They suggest that re-expression of caveolin-1 inhibits the Ras/MAPK pathway and therefore inhibits anchorage-independent growth, and in the case of v-Abl-transformed cells also causes cell death when colonies reach a certain size.

Zhang *et al.* [88] found that motile mammary adenocarcinoma cells possessed reduced levels of caveolin-1, and that expression of caveolin-1 in these cells using an adenovirus vector blocked anchorage-independent growth. It also blocked EGF-stimulated Erk activation, lamellipod extension and cell migration. In a study by Fiucci *et al.* [178], transfecting MCF-7 human breast carcinoma cells (which usually lack caveolin-1) with wild-type caveolin-1 inhibited proliferation, anchorage-independent growth and invasion of the extracellular matrix. It also prevented activation of Erk1/2 on contact with laminin. Lee *et al.* [179] found caveolin-1 to be down-regulated in several breast cancer cell lines, and found that re-expression of caveolin-1 inhibited growth. Finally, Bender *et al.* [180] demonstrated that re-expression of caveolin-1 in colon carcinoma cell lines in most cases reduced their ability to form tumours in nude mice. However, in 30% of cases tumour formation was not reduced, and in these cases it was found that tumour formation resulted in the selection of cells that did not contain caveolin-1.

Targeted Down-Regulation of Caveolin-1 Promotes Cellular Transformation

Galbiati *et al.* [87] used caveolin-1 antisense cDNA to down-regulate caveolin-1 in NIH 3T3 cells. These cells displayed altered morphology, anchorage-independent growth and loss of contact inhibition, all hallmarks of transformation, and were also capable of forming tumours in immunodeficient mice. It was shown that components of the p42/44 MAPK cascade (MEK and Erk) were constitutively active in these cells, and this was necessary for maintenance of the transformed phenotype. In contrast, the p38 MAPK and JNK cascades were not affected. Jasmin *et al.* found that mechanical down-regulation of caveolin-1 in ischemic brain induce impaired angiogenesis and increased apoptotic cell death, whereas the phenotype of caveolin-2 absence was comparable to the wild-type [181].

Caveolin Knockout Mice

Caveolin-1 knockout mice were first reported in 2001, and a number of observations regarding these mice support a role for caveolae in suppressing growth. Razani *et al.* [182] found that embryonic fibroblasts derived from the knockouts proliferated twice as fast as the wild type cells, but this was not due to hyperactivation of the MAPK cascade. Zhao *et al.* also examined knockout mice and noted that they are completeli devoid of caveolae [183]. With the major transcytotic organelle absent, these mice were also predicted to have decreased vascular permeability [183, 184]. The further report of an increase in vascular permeability from Schubert *et al.* was therefore quite surprising [185].

Lin *et al.* [186] also presented evidence that in caveolin-1 null mice there is an increased tumor permeability (defined by the extravasation of Evans blue and deposition of fibrinogen). Tumor permeability and angiogenesis are most likely interdependent, creating a positive feedback to support increased tumor growth. The authors observed a hyperphosphorylation of VEGFR-2 that could be responsible for the increase in tumor permeability and angiogenesis through activation of several pathways.

Cohen *et al.* [187] examined the hearts of caveolin-1 knockouts and found that they possessed significantly thicker left ventricular walls than normal mice, as well as being significantly heavier overall. Closer examination revealed cardiac myocyte hypertrophy (overgrowth) and fibrosis (formation of fibrous tissue). Erk1/2 was hyperactivated in heart tissue and in isolated cardiac fibroblasts. The levels of cyclin D1 (a regulator of the cell cycle) were not altered, although elevated levels of inducible and endothelial nitric oxide synthase may have contributed to the cardiac hypertrophy. In contrast, hyperactivation of Erk1/2 was not detected in caveolin-2 knockout mice.

Lee *et al.* [176] also examined caveolin-1 knockout mice, and found that although these mice failed to spontaneously develop mammary tumours, their mammary glands had an epithelial cell layer several cells thick. In addition, Capozza *et al.* [188] found that caveolin-1 knockout mice were more susceptible to skin carcinogenesis induced by a known carcinogen, 7,12-dimethylbenzanthracene (DMBA).

Williams *et al.* [189] interbred caveolin-1 knockout mice with tumour-prone transgenic mice that normally develop multifocal mammary lesions. In tumour prone mice lacking caveolin-1, the frequency and size of the lesions were greatly increased, while this was not true for mice heterozygous for caveolin-1. However, in this case it was found that cyclin D1 was up-regulated in the mammary lesions of the caveolin-1 null mice as compared to the normal tumour prone mice, but no change in the activation state of the p42/44 MAPK cascade was observed.

Regarding caveolin-2 there are not really clear evidences about a possible role that it plays in cancer. In fact, lack of caveolin-2 does not affect the presence of caveolae, the expression of caveolin-1 and therefore of the receptors involved in caveolae. But still Schubert *et al*. had evidence about age-related skeletal muscle abnormalities [190].

Regarding caveolin-3 knock-out mice, the only evident phenotype is a disfunction in heart. Woodman *et al.* [191] had evidence of hyperactivation of p42/p44 MAPK (Erk1/2) that plays an important role as an effector of the cardiac hypertrophic response. A cardiomyopathic phenotype was also present in the case of caveolin-1 and caveolin-3 double knock-out mice [192].

How is the Expression of Caveolin-1 Down-Regulated in Transformed Cells?

Several studies have investigated caveolin-1 down-regulation in cancer, and hence provided further evidence that it acts as a tumour suppressor. Firstly, Engelman *et al*. [193] showed that caveolin-1 and caveolin-2 are localised in the q31.1 region of chromosome 7, a region that is commonly deleted in human cancers. In addition, caveolin-1 mutations have now been detected: Hayashi *et al*. [194] identified heterozygous mutations in codon 132 (P132L) of caveolin-1 in 16% of human primary breast tumours examined, while failing to observe this mutation in test subjects. Cancers harbouring the mutation were mainly invasive cancers. NIH 3T3 cells transfected with the caveolin-1 mutant showed disruption of the actin cytoskeleton and abnormal morphology, increased growth on soft agar, and constitutive activation of MAP kinases. Transfected cells also demonstrated higher motility and invasive capacity than parental cells or cells transfected with wild type caveolin-1. Lee *et al.* [195] examined the P132L mutation further, and found that, in cells transfected with the mutant protein, the mutant was excluded from low density, Triton X-100 insoluble fractions. Immunofluorescence studies showed that the mutant caveolin-1 was in fact retained at the Golgi complex. They also showed that the mutant protein acts in a dominant negative manner, causing wild-type protein to also be retained at the Golgi complex; this is an important observation, as the mutation was generally found to be heterozygous in breast cancer cells [194].

Hypermethylation of the caveolin-1 promoter may also be a mechanism by which its expression is altered in cancer. Methylation of CpG islands in the promoters of tumour suppressor genes has been observed in transformed cells [196]. Engelman *et al.* found that CpG islands in the caveolin-1 promoter were hypermethylated in two breast cancer cell lines that do not express caveolin-1. Cui *et al.* [197] also noted that caveolin-1 was hypermethylated at CpG islands in its promoter region in prostate cancer cells. However as yet it has not been proven that this methylation leads to down-regulation of caveolin-1, and in fact some of the cancer cells studied by Cui *et al.* [197] actually had elevated levels of caveolin-1. Nevertheless, Wiechen *et al.* [198] did observe that the down-regulation of caveolin-1 in two ovarian cancer cell lines required DNA methylation.

Engelman *et al.* [199] found that caveolin-1 was down-regulated in cells transformed by mutationally activated Ras or Raf, and that treatment with a MEK inhibitor restored caveolin-1 mRNA and protein levels. They suggest therefore that caveolin-1 can be down-regulated by activation of the Ras/MAPK cascade. However, they also found that there were MAPK-independent pathways that could act to down-regulate caveolin-1, for example in v-Src transformed cells. Control of expression appeared to be at the transcriptional level; indeed, transient transfection of CHO cells with activated Raf or Erk down-regulated caveolin-1 promoter activity. Overexpression of protein kinase A was also capable of down-regulating caveolin-1 expression. These results suggest that caveolin-1 can be down-regulated by an oncogenic stimulus such as hyperactivation of the MAPK cascade. Engelman *et al.* [177] also demonstrated that caveolin-1 levels could be regulated by an oncogenic stimulus: they used an NIH 3T3 cell line expressing a temperature-sensitive form of v-Abl. When the kinase was active, caveolin-1 was down-regulated, but at non-permissive temperatures, when the kinase was inactive, caveolin-1 levels returned to those seen in normal cells. They also found that treatment with an inhibitor of MEK led to up-regulation of caveolin-1 (inhibitors of the p38 MAPK pathway, which involves different components, did not affect caveolin-1 levels). Treatment with PDGF or FGF has also been shown to down-regulate caveolin-1 expression in NIH 3T3 cells, and in the latter case at least this was at least partly due to activation of the p42/44 MAPK cascade [87].

Engelman *et al.* [84] examined the relationship between ErbB2 (Neu, HER2) tyrosine kinase activity and caveolin-1 expression. As mentioned previously, the gene encoding ErbB2 (*Neu* or c-*erbB2*) is a proto-oncogene, the overexpression of which can cause cancer in humans [200]. In addition, mutationally activated forms of ErbB2 have been identified in rats and mice: e.g., oncogenic NeuT, the rat homologue of ErbB2, has an activating mutation in the transmembrane region [200]. Engelman *et al.* [84] transfected fibroblast cell lines with various mutated forms of ErbB2 (including NeuT) that are known to have oncogenic potential. Caveolin-1 mRNA and protein were down-regulated in these cells, but not in cells overexpressing wild-type ErbB2. The down-regulation of caveolin-1 by mutationally activated ErbB2 was partly dependent on the activation of the p42/44 MAPK pathway by the receptor. In addition, caveolin-1 inhibited signal transduction from overexpressed wild-type ErbB2 and NeuT, and this inhibition was mediated by the scaffolding domain. In mammary tumours induced in mice by targeted overexpression of ErbB2, caveolin-1 was also down-regulated, as it was in tumours induced by overexpression of the downstream molecules Ras and Src. These results indicate a reciprocal interaction between ErbB2 and caveolin-1.

Caveolin-1 Levels are Not Always Reduced in Transformed Cells

In some tumours and tumour cell lines, it has been found that either caveolin-1 levels are unchanged compared to normal cells, or they are actually increased. For example, while Yang *et al.* [201] found caveolin-1 mRNA levels to be reduced in transformed NIH 3T3 cells, consistent with results discussed above, they also reported elevated caveolin-1 mRNA and protein levels in prostate cancer cell lines derived from metastases. The same was true for tissue samples: normal prostate expressed very low levels of caveolin-1,

which was up-regulated in primary tumours and further up-regulated in metastatic tumours. Increased caveolin-1 expression was also detected in breast carcinoma tissue samples. In addition, Ho *et al.* [202] found that while caveolin-1 levels were low in poorly invasive lung adenocarcinoma cells, caveolin-1 was abundant in highly invasive cells. Caveolin-1 levels were also low in tissue samples from primary lung adenocarcinomas, while caveolin-1 levels were generally higher in metastatic tumours. Furthermore, introduction of caveolin-1 into poorly invasive cells induced filopodia formation and increased the invasive capacity of the cells. Taken together, these results suggest that high caveolin-1 levels correlate with metastasis; this is also consistent with the findings of Bender *et al.* [180], who found that caveolin-1 was up-regulated in a metastatic cell line compared to the parental cell line.

Lavie *et al.* [203] discovered that caveolin-1 was up-regulated in multidrug-resistant colon adenocarcinoma and breast carcinoma cells compared to the parental cell lines. The glycosphingolipid glucosylceramide, a constituent of caveolae, was also up-regulated in multidrug-resistant cell lines, as was caveolin-2. Consistent with these observations, the number of caveolae was increased in multidrug-resistant cells. The authors therefore suggest that caveolae play a role in the development of the multidrug-resistant phenotype. Consistent with results reported above, however, these cells were shown to have a reduced rate of proliferation.

Further results include those of Wiechen *et al.* [198], who also found caveolin-1 to be up-regulated in 11 out of 15 tumour samples from the kidney, prostate and stomach, while Wiechen *et al.* [162] found caveolin-1 levels to be high in both normal mesenchymal tissues and benign mesenchymal tumours. Caveolin-1 has also been shown to be present in T-cell leukaemia cell lines, though not present in normal T-cells [204], and Hurlstone *et al.* [205] found caveolin-1 to be expressed in tumours derived from breast myoepithelium, as well as in normal epithelium. As discussed above, Podar *et al.* [127] found that caveolin-1 was expressed in multiple myeloma cells, and suggest that caveolae have a role in IGF-1R signalling in these cells. However, their experiments do not distinguish between lipid rafts and caveolae.

Interestingly, although Fiucci *et al.* [178] found that expression of caveolin-1 in MCF-7 cells in general inhibited growth and invasion of the cancer cells, these cells were also resistant to anoikis (apoptosis induced by detachment from the extracellular matrix). Based on this observation, they suggest that where cancer cells express caveolin-1, they may have been positively selected due to its negative regulatory effects on apoptosis.

In summary, there is quite a large amount of evidence supporting a role for caveolin-1 as a tumour suppressor gene, including its location at a site commonly deleted in cancers, the identification of mutations in the caveolin-1 gene in some cancers, and its down-regulation in many tumours and tumour cell lines. It could be argued that the use of cell culture systems in many of the papers discussed makes the results less accurate, as conditions used in culture, such as the availability of cholesterol, could affect caveolin-1 expression. However, caveolin-1 has also been found to be down-regulated in tumours themselves, and several independent studies have shown that the re-expression of caveolin-1 in transformed cells inhibits features of the transformed phenotype, such as anchorage-independent growth. Caveolin-1 knockout mice also support the hypothesis, as they display hyperproliferative disorders and hyperactivation of the MAPK cascade, and are at greater risk from carcinogen-induced tumorigenesis [188]. Although these mice do not spontaneously form tumours, knockouts of important cell cycle control agents, such as the cyclin-dependent kinase inhibitor p21, also do not form tumours [206]. Possibly compensatory proteins are involved, but it does not seem that caveolin-3 is one of these, as Razani *et al.* [182] found its levels unchanged in caveolin-1 knockouts. Fig. (**3**) illustrates how loss of caveolin-1 could lead to cellular transformation: caveolin-1 usually inhibits components of the MAPK cascade and growth factor receptors, and so its loss leads to an increased availability of receptors and signalling molecules, causing hyperproliferation. However, although there is evidence that this pathway plays a role in tumour suppressor activity [87] caveolin-1 also has other effects on the cell cycle. For example, it has been shown to repress the transcription of cyclin D1, which is required for cell cycle progression [207], and increased cyclin D1 expression was shown to be the main reason for the increased tumour formation in tumour prone caveolin-1 knockout mice [189]. In addition, caveolin-1 has been shown to cause cell cycle arrest at the G_0/G_1 phase *via* a p53/p21-dependent mechanism [208]. Therefore it is likely that multiple functions contribute to the role of caveolin-1 as a tumour suppressor gene.

The ability of caveolin-1 to negatively regulate growth factor signalling pathways and cell cycle progression, and its identification as a candidate tumour suppressor, might suggest the possibility of cancer treatments based on caveolin-1 or its scaffolding domain. However, the fact that caveolin-1 is up-regulated in some cancers, and especially in metastatic and multi-drug resistant cells, implies that caution would be required here, and clearly further work is required to enable a full understanding as to why caveolin-1 has these effects, and why it is down-regulated in some cancer cells and not others.

CONCLUSIONS AND FUTURE PERSPECTIVES

The evidence presented in this article supports a role for lipid rafts and caveolae in the regulation of signalling from growth factor receptors. For many of the receptors it is not yet possible to say what the exact roles of each domain are, and further work is required to give a better understanding of the situation. However, caveolae are capable of inhibiting several receptors including the EGFR, TrkA and PDGFR, as well as components of the MAPK pathway (although possibly not the PI3K/Akt pathway). In keeping with this, caveolin-1 seems to act as a tumour suppressor in some cell types. In other cells, however, it is actually up-regulated upon transformation, and it also appears to be re-expressed in metastatic tumours. This suggests that other factors are involved, some of which are cell type specific.

A model was presented here to suggest that lipid rafts have an activatory role in EGFR signalling, forming signalling platforms that allow efficient signal initiation and propagation. Such a role for lipid rafts is also possible in signalling by other receptors. Furthermore, this indicates a possible role for lipid rafts in cancer, and it has been sug-

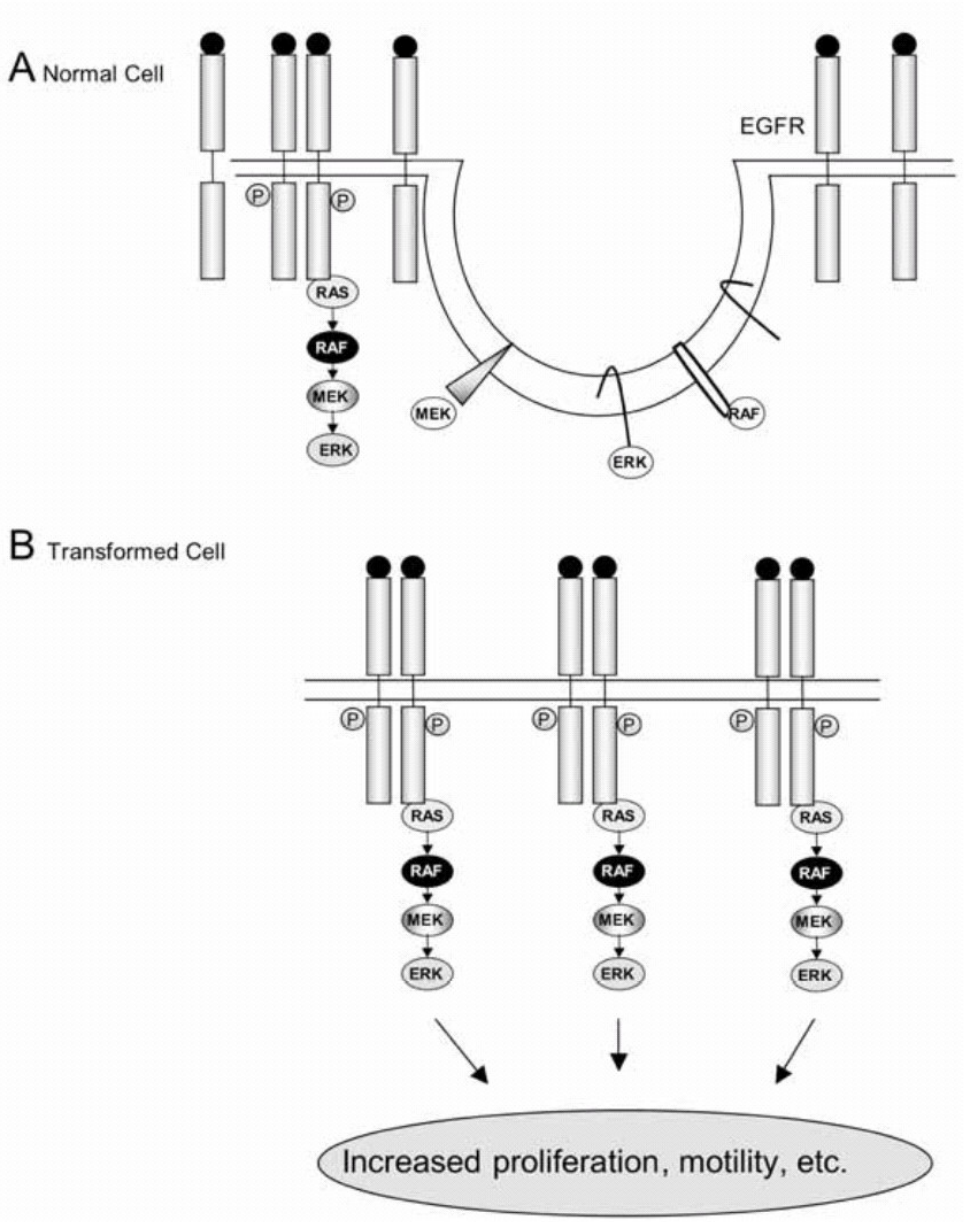

Fig. (3). Model for the role of caveolae in cellular transformation. A) In a normal cell, caveolin (hairpin structures) binds and inhibits receptors e.g., the EGFR and components of the MAPK cascade. **B**) In a transformed cell with low caveolin levels, more growth factor receptors and components of the MAPK cascade are free from the inhibitory effects, and this leads to increased proliferation. For clarity, not all components of the MAPK cascade are shown. Inactive molecules are shown as open ovals, whereas active molecules are depicted as filled ovals; EGF = epidermal growth factor; Erk = extracellular signal-regulated kinase.

gested that this might be the basis of a link between cholesterol intake and prostate cancer; i.e. that increased cholesterol increases raft size, stabilises EGFR signalling and increases cell growth and survival.

The proposed involvement of lipid rafts and caveolae in cancer suggests the possibility of treatments that target these domains. However, a greater understanding of the reasons why caveolin-1 is up-regulated in some metastatic and drug-resistant cells, and why it only acts as a tumour suppressor in some cancers, is first required. This would be necessary before the development of any treatment based on caveolin-1 or its scaffolding domain, which inhibits components of the MAPK cascade. If such treatments were found to be possible, they would be very specific for certain cancers. The same is true for any attempt to treat cancer by cholesterol

depletion, and in this case *in vivo* studies are needed to determine the exact link between cholesterol levels and cancer, and to confirm the hypothesis that increased cholesterol in the plasma membrane increases EGFR signalling.

It is clear to see that reports regarding the protein composition of lipid rafts and caveolae can often be contradictory. In many cases this is due in part to the fact that it is difficult to isolate these domains separately. In addition, there is the problem of detergent insolubility. In some cases, for example for the EGFR, proteins are found to be soluble in one detergent such as Triton X-100, and insoluble in another such as Brij 58. As yet, the reasons for this are unclear: as explained above, it has been suggested that it may reflect a certain type of association of a protein with rafts, or possibly may indicate the presence of distinct types of lipid raft. The latter possibility is intriguing, as it could be that different types of domain mediate signal transduction from different receptors, and this is worth further investigation. Regarding different types of caveolae, two forms, 'deep' and 'shallow' have been identified, as explained in the introductory section, and again more work is required to determine if they have functional differences.

In the future, experiments on lipid rafts and caveolae will be aided by the use of techniques which allow a more accurate study of these domains [35]. At present, some of the major methods used include cholesterol depletion, which can have many effects on a cell, and the use of detergents, which have unpredictable effects on certain proteins. Because of their small size and their nature as integral components of cell membranes, lipid rafts and caveolae are still difficult to study, and this has been a hindrance to the study of their functions in signal transduction.

ABBREVIATIONS

CREB = cAMP response element-binding protein

DMBA = 7,12-dimethylbenzanthracene

EGFR = Epidermal growth factor receptor

Erk = Extracellular signal-regulated kinase

FGF = Fibroblast growth factor

FRET = Fluorescence resonance energy transfer

FRS2 = Fibroblast growth factor receptor substrate 2

GPI = Glycosylphosphatidylinositol

IGF-1R = Insulin-like growth factor-1 receptor

IP_3 = Inositol 1,3,5-trisphosphate

JNK = c-Jun N-terminal kinase

MAPK = Mitogen-activated protein kinase

MEK = Mitogen-activated Erk kinase

mTOR = Mammalian target of rapamycin

NGF = Nerve growth factor

PA = Phosphatidic acid

PDGFR = Platelet-derived growth factor receptor

PDK1 = 3-phosphoinositide-dependent kinase-1

PI3K = Phosphatidylinostol 3-kinase

PIP_2 = Phosphatidylinositol-4,5-bisphosphate

$PI(3,4)P_2$ = Phosphatidylinositol-3,4-bisphosphate

PIP_3 = Phosphatidylinositol-3,4,5,-trisphosphate

PKC = Protein kinase C

PLC = Phospholipase C

PLD = Phospholipase D

S6K1 = Ribosomal protein S6 kinase 1

SH2 = Src homology region 2

SHIP = SH2-containing inositol-5-phosphatase

Trk = Tyrosine kinase receptor

uPAR = Urokinase-type plasminogen activator receptor

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5.2 Recent Patent of Gene Sequences Relative to the Phosphatidylinositol 3-kinase/Akt Pathway and their Relevance to Drug Discovery

Recent Patents on DNA & Gene Sequences 2007, *1*, 000-000 1

Recent Patents of Gene Sequences Relative to the Phosphatidylinositol 3-kinase / Akt Pathway and their Relevance to Drug Discovery

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Abstract: Phosphoinositide 3-kinases (PI3Ks) play an essential role in the signal transduction events initiated by the binding of extracellular signals to their cell surface receptors. There are eight known PI3Ks in humans, which have been subdivided into three classes (I-III). The class I_A of PI3K comprises the p110α, p110β and p110δ isoforms, which associate with receptor tyrosine kinases (RTKs). On the other hand, the class I_B PI3K p110γ is regulated by G-protein-coupled receptors (GPCRs). Gene targeting studies in mice have revealed specific biological functions for the class I_A p110δ in lymphocyte activation, and the class I_B p110γ in inflammatory cell responses. In human cancer, recent reports have described activating mutations in the *PIK3CA* gene encoding p110α, and inactivating mutation in the *PTEN* gene, a tumor suppressor and antagonist of the PI3K pathway. Thus, individual PI3K isoform are potential drug targets for a variety of human diseases, including allergies, cancer, rheumatoid arthritis and arterial thrombosis. In this review, we will discuss recent patents relating to class I PI3Ks, including patents on the cDNA sequences of p110γ and p110δ. Moreover, we will review patents on novel pharmacological PI3K inhibitors and on methods of manipulating T cell responses through PI3K.

Keywords: phosphatidylinositol 3-kinase, Akt, receptor tyrosine kinase, G-protein-coupled receptor, cancer, inflammation, rheumatoid arthritis, allergy

INTRODUCTION

PHOSPHOINOSITIDE 3-KINASES

The phosphoinositide 3-kinases (PI3K) are a family of evolutionary conserved lipid kinases, which play a crucial role in controlling a wide variety of intracellular signaling events. PI3Ks phosphorylate phosphatidylinositol (PI) on the D-3 position of the inositol ring, producing distinct second messengers such as PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 (PIP_3) [1]. These second messengers are known to activate diverse target proteins involved in complex signaling cascades, ultimately resulting in the activation of cellular responses including growth, proliferation, survival and motility.

The family of PI3Ks identified in various species can be subdivided into three main classes (class I-III), based on structural similarity and *in vitro* substrate specificity [1] (overview given in Fig. (**1**)). Class I_A includes p110α, p110β, and p110δ, which are known to form a heterodimeric complex with a p85, p55, or p50 (α or β) regulatory subunit. This adapter subunit contains two Src-homology 2 (SH2) domains mediating their association with activated tyrosine kinase-coupled growth factor receptors [1]. PIP_3 produced by class I_A PI3Ks activates the protein kinase phosphoinositide-dependent protein kinase-1 (PDK1), inducing the recruitment and activation of the key signal transducer protein kinase B (PKB)/Akt [2]. Akt is involved in regulation of the cell cycle and glucose metabolism through glycogen synthase kinase-3 (GSK3) [3] and in modulation of cell growth and survival. Moreover, Akt controls the translational machinery through the mammalian target of rapamycin (mTOR), the ribosomal protein S6 kinase (S6K) and the 4E-binding protein (4E-BP) [4]. Downstream events controlled by Akt further include the control of apoptosis through the regulation of proteins such as forkhead (FKHR), BAD, NF-κB and murine double minute gene-2 (MDM-2) [5] (overview given in Fig. (**2**)). Expression studies of the class I_A PI3K isoforms have revealed an ubiquitous distribution of the subunits p110α and p110β in human tissues, whereas p110δ appears to be selectively expressed in leukocytes [6-8].

The class I_B of PI3Ks is composed of one enzyme only, p110γ, which functions through heterodimer formation with a regulatory subunit p101 [9] or p84 [10]. Activation of p110γ is controlled by receptors capable of activating heterotrimeric guanine nucleotide-binding proteins, termed G-protein coupled receptors (GPCRs) [9]. The PI3K p110γ is thought to link GPCR signaling to PIP_3 production, which controls cell motility in inflammatory cells such as macrophages and neutrophils [11] (Fig. (**3**)).

The human class II of PI3Ks comprises the three isoforms PI3KC2α, PI3KC2β and PI3KC2γ [1]. The hallmarks of class II family members are a substrate specificity restricted to PI and PI(4)P *in vitro* and a C-terminal C2 domain. Although the precise cellular function of these enzymes remains generally poorly understood, recent reports have described class II PI3Ks as downstream transducers of activated polypeptide growth factor receptors [12, 13]. The

*Address correspondence to this author at the Division of Clinical Chemistry and Biochemistry, University Children's Hospital Zurich, Steinwiesstrasse 75, CH-8032 Zurich, Switzerland; Tel: +41 44 266 7640; Fax: +41 44 266 7169; E-mail: Alexandre.Arcaro@kispi.unizh.ch

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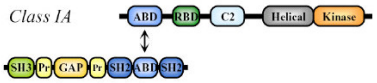
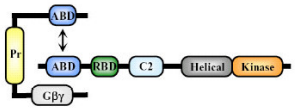
Class I	Subunits Regulatory	Subunits Catalytic	Substrate Specifity	Activator	Tissue Distribution
Class IA 	p85α p85β p55α p55γ p50α	p110α p110β p110δ	PI PIP PIP_2	Receptor tyrosine kinases Ras	p110α, p110β: ubiquitous p110δ: leukocytes
Class IB 	p101 p84	p110γ	PI PIP PIP_2	G-protein-coupled receptors (Gβγ) Ras	leukocytes
Class II					
		PI3KC2α,β,γ	PI PIP	Receptor tyrosine kinases G-protein-coupled receptors	PI3KC2α,C2β: ubiquitous PI3KC2γ: liver
Class III					
	p150	Vps34p analogues	PI	Constitutively active G-protein-coupled receptors (Gα)	

ABD – Adaptor Binding Domain
RBD – Ras Binding Domain
C2 – C2 Domain
Helical – Helical Domain
Kinase – Kinase Domain
SH3 – Src Homology Type 3 Domain
Pr – Proline-rich Domain
GAP – Bcr/Rac GAP Homology Domain
SH2 – Src Homology Type 2 Domain
Gβγ – Gβγ-binding Site

Fig. (1). Overview of PI3K isoforms. The PI3K family consists of eight isoforms, which are grouped into three classes, based on sequence homology and *in vitro* substrate specificity. The class I isoforms are further subdivided into class I_A and class I_B isoforms based on their mechanism of action. While certain PI3K isoforms are expressed ubiquitously, isoforms such as p110γ or p110δ have been found to be more tissue-specific.

class III of PI3K includes a homolog of the yeast vesicular protein-sorting protein Vsp34 [14] and its major function is in intracellular trafficking events [15].

INHIBITION OF PI3K SIGNALING

As PI3Ks have been identified as playing critical roles in distinct cellular signaling processes, a precise understanding of these kinases, their substrates and effectors is of particular interest. Anomalies in signaling cascades have been described in various human diseases and thus a better understanding of these events is of high importance in the search for therapeutic, diagnostic and screening applications. A number of attempts have been made to better understand PI3Ks in general, as well as to gain insight into the specific functions of the different isoforms. The first selective pharmacological PI3K inhibitors to have been described are wortmannin [16, 17], a compound that was originally isolated from soil bacteria and is toxic to fungi, and LY294002 [18], a morpholino derivative of quercetin, a naturally occurring bioflavinoid and broad spectrum kinase inhibitor. Both compounds have been shown to inhibit cell growth at concentrations that would be expected to inhibit class I PI3Ks. However, as these pharmacological inhibitors display little selectivity within the PI3K family and might also affect other kinases, further research has been aimed at developing compounds with improved specificity and pharmacokinetic properties. Various PI3K inhibitor prodrugs and their possible pharmaceutical applications have been patented by Garlich *et al.* [19]. In addition, patents exist on various PI3K polypeptides, nucleic acid sequences encoding PI3Ks, antibodies that are specifically immunoreactive with PI3Ks and methods for using these reagents in screening and therapeutic applications.

GENE-TARGETING STRATEGIES

Gene-targeting strategies have been aimed at deleting specific PI3K isoforms and have uncovered key roles of the different enzymes in immunity, metabolism, cardiac function, cell proliferation and cancer susceptibility. Different knock-out mice with targeted deletions of genes encoding PI3K regulatory and catalytic subunits have been generated and phenotypically analyzed [20].

A knock-out mouse with a deletion in the gene of the PI3K class I_A catalytic subunit p110α has been generated by

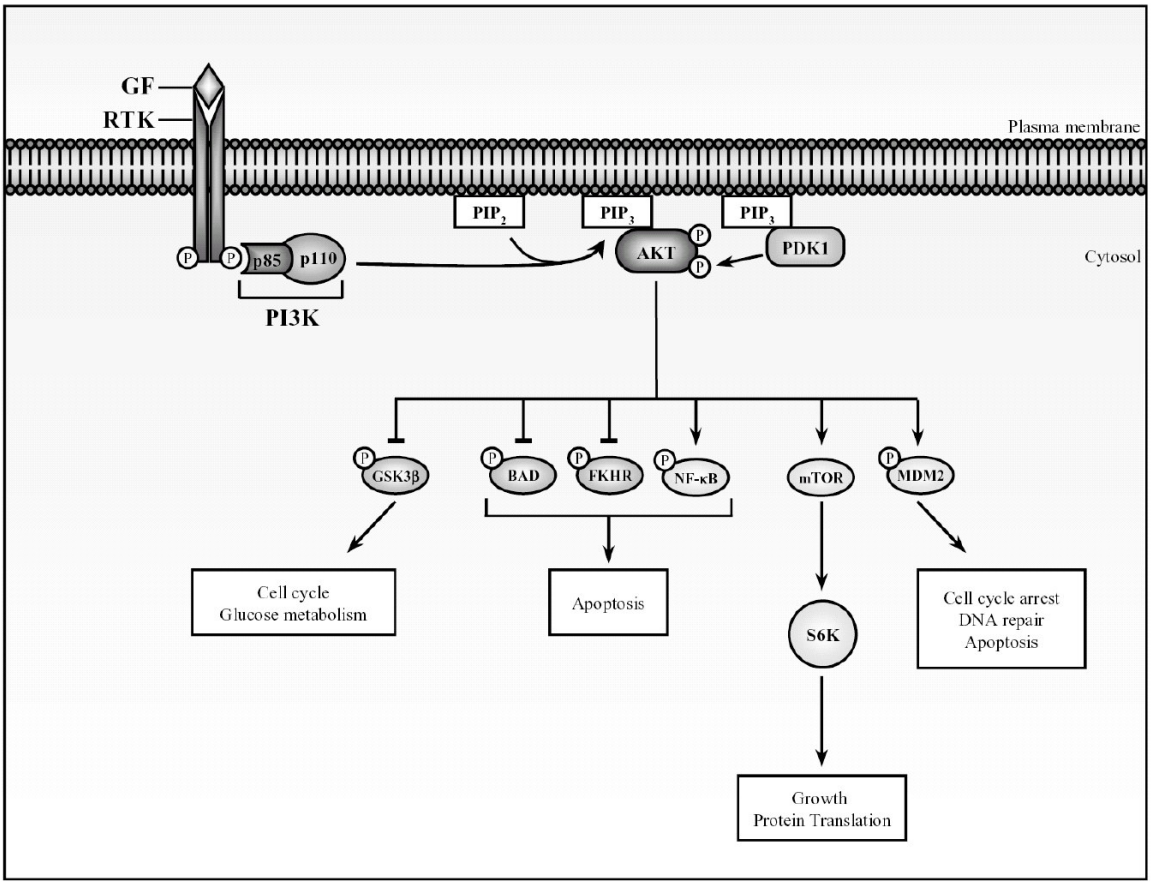


Fig. (2). Overview of the phosphoinositide 3-kinase (PI3K) signaling pathway. Upon growth factor (GF) binding, transmembrane receptor tyrosine kinases (RTK) are activated by autophosphorylation. PI3K activation occurs when the regulatory p85 subunit is recruited to specific phosphotyrosine sites located in the intracellular domain of the activated RTK. Key downstream signaling mediators include phosphoinositide-dependent kinase 1 (PDK1) which is essential for activation of protein kinase B (PKB/Akt). Effector molecules include glycogen synthase kinase-3 (GSK-3), BAD, forkhead (FKHR), nuclear factor kappa B (NF-κB), mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase (S6K) and murine double minute gene 2 (MDM2).

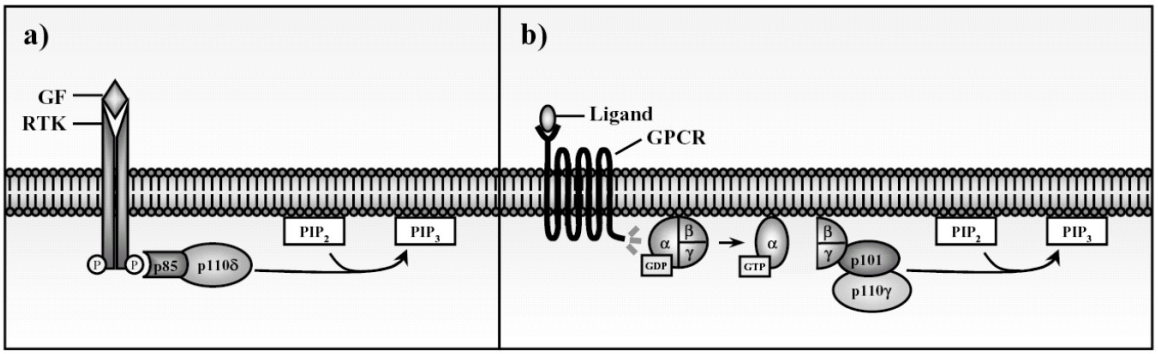


Fig. (3). Activation of p85/p110δ and p101/p110γ occurs via different receptors. While the p85/p110δ complex is recruited to activated receptor tyrosine kinases (RTK) following growth factor (GF) binding (a), the p101/p110γ complex is activated by the Gβγ subunit following binding of a specific ligand to G protein-coupled receptors (GPCR) (b).

targeting the p85-binding domain of the *PIK3CA* gene, leading to loss of expression of this specific isoform [21]. The $PIK3CA^{del/del}$ embryos showed a clear developmental delay and died between days 9.5 and 10.5 of embryonic development [21]. In embryos missing the p110α enzyme, a profound proliferation defect could be observed, which was further supported by a failure in replication of p110α-deficient fibroblasts in culture medium, even when supplemented with growth factors. The developmental period between E9.5 and E10.5 is known for increased cellular proliferation, growth, and differentiation. It has thus been hypothesized that the intrauterine death of $PIK3CA^{del/del}$ embryos at this stage was caused by the incapacity to provide the increased demand in proliferative signals maintained by PI3K signaling through the p110α isoform [21]. Moreover, mice deficient in the p110α isoform displayed multiple vascular defects [22]. A role for p110α in the control of cell growth was further highlighted by an increase in heart size in transgenic mice expressing a constitutively active mutant of p110α [23]. Conversely, a decrease in heart size was observed upon expression of a dominant-negative mutant of this PI3K isoform in the heart [23]. The interpretation of knock-out data, however, has been complicated by the observation of an upregulation of other PI3K subunits after the deletion of one specific isoform. In $PIK3CA^{del/del}$ homozygous embryos there was an apparent increase in the expression of the PI3K class I_A regulatory subunit p85/p55, raising the question of a contributing phenotypical effect by these adapter proteins. Further insight into the function of p110α has been given by the generation of mice carrying a knock-in mutation (D933A) that abrogates the p110α kinase activity [24]. Homozygocity for this mutation resulted in embryonic lethality, while heterozygocity led to impaired signaling via the insulin-receptor substrate (IRS) proteins, which are key mediators of insulin, insulin-like growth factors I (IGF-I) and leptin action. As a result, the mutant mice displayed reduced somatic growth, hyper-insulinaemia, glucose intolerance, hyperphagia, and increased adiposity. Another study recently defined the p110α subunit as the critical lipid kinase required for insulin signaling in adipocytes and myotubes [25]. The discovery of the role of p110α as a key intermediate in metabolic signaling raises concerns about potential side-effects of PI3K inhibitors .

The biological functions of the PI3K class I_A catalytic subunit p110β have been studied by a partial deletion allele knock-out mouse [26]. Targeting of this specific isoform resulted in very early embryonic lethality at the homozygous state. Zygotes with the $PIK3CB^{del/del}$ genotype were non-viable very early after fertilization, leading to a deficiency at the blastocyst stage [26]. Crossbreeding studies of p110β knock-out mice with p110α-deleted mice did not reveal any possible redundant functions of these two class I_A catalytic isoforms. However, a possible overlap in functions that might be manifested in more subtle phenotypical abnormalities could not be ruled out so far [26].

Mice deficient in the class I_A PI3K catalytic subunit p110δ have been described by three different groups [27-29]. These studies revealed a major function of p110δ in B and T lymphocytes and a failure of the knock-out mice to mount normal immune responses. The mice lacking p110δ showed a reduction in the amount of B1 and marginal zone B cells, as well as lowered serum levels of immunoglobulins [27]. Furthermore, antigen receptor signaling in B and T cells was impaired and the mice were prone to the development of inflammatory bowel disease [28]. However, despite the high levels of p110δ expression in hematopoietic tissues, no significant differences were found in either the number and morphology of erythrocytes, peripheral leukocytes and lymphocytes, or hemoglobin levels between wild-type and p110δ-deficient mice [29].

Knock-out mice with a targeted deletion of the PI3K class I_B catalytic subunit p110γ have been characterized by three different groups [11, 30, 31]. These studies described phenotypes mainly in components of the innate immune response. Mice lacking p110γ showed an accumulation of defective neutrophils, which exhibited a failure in their migratory capacity and a reduced thymic cellularity. Moreover, chemoattractant-stimulated signal transduction was inhibited, because of an impairment of PIP_3 production in p110γ-deficient cells [30]. In contrast to p110δ, the deletion of p110γ resulted in no effect on B cells. Instead, this isoform appeared to regulate proliferation and cytokine production of T lymphocytes [30]. In view of the impaired migration capacity of p110γ knock-out macrophages towards a wide range of chemotactic stimuli, a crucial role for macrophage accumulation in the inflammatory response was furthermore suggested [11]. Phenotypical analysis of mice expressing a kinase-dead p110γ only partially reproduced the phenotype of knock-out animals [32]. Whereas both mice exhibited an identical impairment in innate immune reactions, the p110γ-deficient mice additionally showed a basal enhancement of cardiac contractility and cardiac tissue damage upon pressure overload. It was therefore suggested that p110γ possesses a kinase-independent function in the control of cardiac responses. A role of p110γ in the control of heart function was also described by Crackower *et al.* [33]. These studies described a role for p110γ as a negative regulator of cardiac contractility through the inhibition of cAMP production.

Regarding the regulatory PI3K subunits, gene-targeting strategies have been aimed at disrupting the adapter subunits p85α, its splice variants p55α and p50α, as well as p85β. Ablation of the whole *PIK3R1* gene, which encodes p85α and the mentioned splice variants, resulted in perinatal lethality [34]. However, disruption of the *p85α* gene only lead to impaired B cell development, a reduction in the number of mature B cells, reduced B cell proliferative responses and a lack of T cell-independent antibody production [35]. Homologous deletion of the gene encoding the adapter subunit p85β resulted in a growth reduction of the knock-out mice, compared to their wild-type littermates [36]. Surprisingly, muscle and adipose tissues of both the p85α and p85β knock-out mice exhibited increased insulin-stimulated PI3K activation, enhanced translocation of the glucose transporter isoform-4 to the plasma membrane, as well as hypoglycemia with decreased plasma insulin [34, 36]. Moreover, the mice showed enlarged muscle fibers, brown fat necrosis and calcification of cardiac tissue [34].

PHOSPHOINOSITIDE 3-KINASES AND HUMAN DISEASE

As the PI3Ks are known to be involved in a wide spectrum of control mechanism in the cell, deregulation of their function has been linked to various human diseases. Key roles of the PI3Ks have so far been described in tumor formation and metastasis, chronic inflammation, allergy and cardiovascular disease.

The importance of PI3K signalling in human cancer is highlighted by the fact that there are numerous oncogenes and tumor suppressor genes whose deregulation activates PI3K signaling and enhances the malignant properties of cells [37]. A prominent finding in this context were mutations in the tumor suppressor gene phosphatase and tensin homologue (*PTEN)*, which have been described in various human tumors [1, 37-39]. PTEN is a phosphatase that antagonizes the action of PI3K by de-phosphorylating the D-3 position of polyphosphoinositides [40]. The DNA sequence copy number of *PIK3CA*, the gene encoding the p110α catalytic subunit of PI3K located on chromosome 3q26, is frequently increased in ovarian cancers [41]. Moreover, recent reports have described activating mutations in the *PIK3CA* gene in a variety of other human cancers, including, breast, colon and medulloblastoma [42, 43]. Although mutational alterations have predominantly been characterized for p110α so far, an increase in p110β activity was also found in human colon cancer biopsies and adenocarcinoma cell lines [44]. Furthermore, a knock-down analysis of p110β in a prostate cancer mouse model suggested a role for this PI3K isoform in the formation of metastasis [45].

Gene-targeting strategies have furthermore uncovered a role p110γ in colorectal adenocarcinoma [46]. Lack of p110γ significantly increased the incidence rate of sponta-neous development of multifocal carcinomas and invasive adenocarcinoma in the colon of mice. In humans, protein expression analysis of p110γ revealed a loss of this protein in a high number of colon cancer cell lines, as well as in primary adenocarcinomas isolated from the colon of human patients [46].

As shown by different studies [30, 47], p110γ also plays a pivotal role in mediating leukocyte chemotaxis and activation, as well as mast cell degranulation, thus suggesting an involvement in inflammatory diseases. A specific contribution of the p110γ PI3K isoform to inflammation was supported by a recent study demonstrating that p110γ-deficient mice were protected from rheumatoid arthritis [48]. These mice were essentially protected against collagen II-specific antibody-induced arthritis, correlating with the defective neutrophil chemotaxis observed in this knock-out model [48]. Moreover, treatment of different rheumatoid arthritis mouse models with orally active small-molecule inhibitors of p110γ suppressed the progression of joint inflammation and damage in these mice [48]. Other studies, however, have described a positive role for the PI3Ks in the inflammatory response. In rheumatoid arthritis, the anti-inflammatory cytokine IL-10 is spontaneously produced by macrophages and infiltrating blood lymphocytes in the rheumatic joint [49]. An involvement of PI3K signaling in the regulation of IL-10 is known [50] and further analysis demonstrated that inhibition of PI3K signaling suppressed the production of this cytokine [51].

The PI3K isoform p110δ has also been shown to be involved in the inflammatory response [27]. Recent studies further supported this finding and described a contribution to allergen-IgE-induced mast cell activation and vascular permeability, which are common characteristics of chronic inflammations, such as asthma [52]. Pharmacological inhibition of p110δ in a murine asthma model resulted in a significant reduction in IgE serum levels and in an attenuation of airway inflammation and hyper-responsiveness, by preventing vascular leakage [52, 53].

Gene knock-out studies of the class I_A regulatory subunits in skeletal muscle of mice revealed a significant reduction in muscle weight and fiber size. Moreover, these mice exhibi-ted insulin resistance in the muscle and whole-body glucose intolerance [54]. The p85 regulatory subunits are therefore thought to act as critical mediators of PI3K signaling in the regulation of muscle growth and metabolism. Furthermore, they are thought to make an important contribution to the symptoms of hyperlipidemia associated with human type 2 diabetes [54]. Diabetes is associated with vascular complica-tions, including the impairment of vascular function and alterations in the reactivity of blood vessels to vasoactive agents [55]. It has been shown that PI3K signaling plays a role in vascular growth, proliferation and apoptosis and is implicated in modulating vascular smooth muscle cell contractility [56]. A recent study demonstrated that selective inhibition of PI3K attenuated the development of diabetes-induced abnormal vascular reactivity in the carotid arteries of diabetic rats [57].

The growing understanding of the biological functions of PI3Ks has opened new insights into the links between signaling events and cellular responses. As a result, these kinases have become interesting targets in clinical research, driving the urge to patent. Patents were deposited on various components involved in, or targeting PI3K signaling, such as polynucleotides encoding PI3Ks [58], constitutively active PI3Ks and uses thereof [59], cDNA sequences of different PI3K isoforms [60, 61], signaling pathway trans-ducers [62], pharmacological inhibitors [63], and various derivatives [64-68].

PATENTS

Cloning, Expression And Characterization Of A Novel Form Of Phosphatidylinositol-3-Kinase [60, 69]

Stoyanov *et al*. reported for the first time the cloning and characterization of a G protein- activated human phosphatidylinositol 3-kinase [70]. Here we will discuss the identification of the novel isoform of the PI3K family, termed p110γ and the cloning, expression and characterization of this enzyme. These findings were patented by the United States Patent Office and by the European Patent Office [60, 69]. The invention concerns a nucleic acid sequence, encoding p110γ, an antibody directed against the protein, and discusses the diagnostic and therapeutic use of the protein, the nucleic acid sequence and the antibody. The invention comprises seven nucleic acid/protein sequences (SEQ ID

NO: 1-7) and eleven claims regarding the use of these sequences.

The major subject matter of the invention is a nucleic acid sequence encoding a novel PI3K isoform, p110γ. In order to isolate the cDNAs, a human bone marrow cDNA library was screened using the polymerase chain reaction. A human bone marrow cDNA library was chosen based on the findings by two other groups, which identified a novel PI3K activity in purified neutrophils and platelets stimulated by G protein βγ subunits [71, 72]. For the library screening, oligonucleotide primers were generated based on the highly conserved amino acid sequences KNGDDLR (termed SEQ ID NO.6) and HIDFG (termed SEQ ID NO.7), which were reported for the catalytic subunit of the first cloned and characterized PI3K (residues 803-809 and 932-936 on p110α) [73]. Using these primers, a 402 bp-long fragment was obtained and subsequently subcloned and sequenced. The PCR product showed a 57% homology to the corresponding region of the gene encoding the bovine p110α. These PCR fragments were further used as a probe for the identification of a set of overlapping clones from a human U937 cDNA library. These overlapping clones were isolated and analysed, which led to the identification of the largest clone, containing the nucleic acid sequence termed by the authors SEQ ID NO.1. This cDNA sequence contained an open reading frame which codes for a protein of 1049 amino acids (termed SEQ ID NO.2). The new protein sequence was termed p110γ and displayed a molecular weight of approximately 120 kDa, similar to the previously published catalytic isoforms p110α and p110β [73, 74]. Furthermore, two other highly similar nucleic (SEQ ID NO.3) and amino acid (SEQ ID NO.4) sequences were obtained from the cDNA library, which code for p110γ with 1050 amino acids. Thus the invention encompasses two nucleic (SEQ ID NO.1,3) and two amino acid (SEQ ID NO. 2,4) sequences coding for human p110γ. It further includes naturally occurring allelic human variations of p110γ, as well as proteins produced by recombinant DNA technology, which correspond to the proteins encoded by these sequences.

Furthermore, sequence homology analysis revealed that the amino acid sequence of p110γ has a homology of 36% to the human p110α, 33.5% to the human p110β and 27.7% to the yeast PI3K Vps34. The 400 C-terminal amino acids comprised a highly conserved region in these PI3Ks, which encodes a putative catalytic domain. The authors reported moreover that there was no significant homology in the amino-terminal regions of these PI3Ks, which in p110α is known to be responsible for the binding to the regulatory subunit p85α and p85β [75]. This finding raised the question of a different regulatory mechanism for p110γ.

A further subject of the invention is the detection of p110γ at the protein and transcriptional level. The availability of an antibody that specifically recognizes epitopes in specific regions of p110γ would be a big advantage for the detection of the protein in biological samples and may be applied in diagnostic techniques, where patient samples could be tested for abnormal levels of this protein. In order to detect the expression of p110γ at the protein level, a polyclonal rabbit antiserum against p110γ was produced by immunization with a 15 amino acid-long peptide corresponding to a unique sequence of p110γ NSQL PESFRVPYDPG (SEQ ID NO.5). The expression of p110γ in mammalian tissues was analyzed by immunoprecipitation with this specific antibody and subsequent Western blot analysis using the same antibody. In the human leukemic cell lines U937 and K562, p110γ was detected as a 110 kDa protein. Hence, a further subject matter of the invention is an antibody specific for p110γ, which does not show any cross-reaction with other PI3Ks.

Furthermore, the invention encompasses the detection of p110γ at the transcriptional level under stringent hybridization conditions. Multi-tissue Northern blot analysis was performed with random prime-labeled PCR fragments encompassing the sequences encoding amino acids 1 to 233 of p110γ. This analysis revealed different expression levels of a 5.3 kb-long mRNA in human tissue from pancreas, kidney, skeletal muscle, liver, lung, placenta, brain and heart.

The invention encompasses further the recombinant expression of p110γ. The cloning of the nucleic acid sequence coding for p110γ in principle allows the construction of an expression vector enabling the production of the p110γ protein in a suitable host cell. The authors presented the use of a baculovirus expression system for the expression of p110γ. The cDNA encoding p110γ was cloned from codon 4 into the pAcG2T baculovirus transfer vector (BD Biosciences), which allows foreign genes to be expressed as glutathione S-transferase (GST) fusion proteins. Sf9 cells were co-transfected with pAcG2T-p110γ and linearized baculovirus DNA prior to expression and purification of the recombinant protein according to standard protocols [75]. This method further allowed the characterization of the substrate specificity of p110γ, which was found to phosphorylate PI, PI(4)P and PI(4,5)P_2 on the D-3 position of the inositol ring and was inhibited by wortmannin at nanomolar concentrations. However, recombinant p110γ failed to bind the p85α or p85β regulatory subunits. Furthermore, the catalytic activity of the recombinant p110γ could be stimulated when incubated with the Gβγ subunits, or by Ras-GTP.

Based on the findings described above, the authors further presented a pharmacological model based on the p110γ protein, the antibody targeting it and the nucleic acid sequence coding for the active protein. These tools could be used for analyzing the expression and modulating the activity of p110γ, which could lead to alterations in cell proliferation, histamine release, differentiation, or glucose transport.

We will now present further insight into the importance of this patent, the increasing evidence of the biological importance of p110γ in human diseases and their treatment with novel specific inhibitors. After the purification of the catalytic p110γ and the regulatory p101 subunit from pig neutrophils [76], the crystallographic structure of the highly conserved catalytic subunit of the porcine p110γ was reported and revealed in detail the structure of the catalytic domain and the Ras-binding domain (RBD) of p110γ [77]. Since pharmacological inhibition of the PI3K activity by wortmannin [16], or LY 294002 [18] was useful to demonstrate their role in a variety of leukocyte responses,

but failed to discriminate between distinct PI3K isoforms, neutralizing, isoform-specific antibodies were used for this purpose. For the first time, such an inhibitory antibody was used to determine the role of p110γ in leukocyte function, which indicated that this enzyme is required for natural killer cell migration upon chemokine stimulation [78]. The fact that p110γ is the sole PI3K isoform activated by GPCRs *in vivo* has been definitively shown in p110γ knockout-mice. Three groups reported the generation of p110γ-deficient mice, which revealed that inactivation of p110γ allows normal embryonic and adult development, but causes defects in the immune system [11,30,31]. p110γ-deficient neutrophils showed defects in migration and oxidative burst in response to GPCR agonists. Furthermore, it was reported that p110γ controls thymocyte survival and activation of mature T cells, but has no role in the development or the function of B cells. Moreover, it was observed that p110γ-deficient microphages have significantly reduced migration capacity towards chemotactic agents [11]. Taken together, these data demonstrate that p110γ has a crucial function in linking GPCR signaling to $PI(3,4,5)P_3$ production and hence controls the motility of neutrophils and macrophages. Since abnormal macrophage infiltration causes chronic inflammatory diseases, Hirsch *et al*. further proposed, that p110γ could be a suitable target for the development of novel specific inhibitors to modulate the function of inflam-matory cells [11]. A subsequent study revealed a novel role for p110γ in the modulation of mast cell activation and the control of allergic and inflammatory responses [47]. Despite the crucial role of mast cells in the response to infectious agents and parasites, abnormal mast cell activation can cause allergy, asthma and ultimately an anaphylactic shock. The release of histamine-containing granules and the increase in intracellular calcium concentration were both attenuated by the loss of p110γ. Furthermore, the existence of an autocrine activation loop leading to enhanced mast cell activation was proposed [47]. These observations pointed out that therapeutic inhibition of p110γ could result in a therapeutic advantage by diminishing the development of chronic inflammatory diseases and the migration of inflammatory cells, since multiple chemokine agonists activate GPCRs which signal through p110γ. Based on the emerging evidence that leukocyte motility is crucial in inflammatory disease and that signal transduction occurs through p110γ, Fuchikami *et al*. presented the first high-throughput *in vitro* screen for p110γ activity. The assay made use of recombinantly expressed His-tagged human p110γ and had the potential for high-throughput screening for inhibitors of lipid kinase activity [79]. Such an assay could be very useful in identifying novel specific p110γ inhibitors, due to fact that the catalytic activity of p110γ is crucial for leukocyte migration. It has indeed been reported that mice expressing catalytically inactive p110γ show the same chemotactic defects as those observed in $p110\gamma^{-/-}$ mice [32]. The application of novel specific p110γ inhibitors identified by improved screening approaches resulted in the successful treatment of two chronic inflammatory diseases: systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) in mice [48, 80]. These results revealed for first time the high efficacy of targeting signaling molecules in chronic inflammatory disorders and arouse hope for developing therapies with diminished side effects and increased life quality for patients with chronic inflammatory diseases. The use of these specific p110γ inhibitors further revealed the contribution of this isoform to thrombus stability [81]. Hence the development of anti-thrombus therapies based on recent findings that p110γ modulates the formation and stability of multi-platelet aggregates will be of interest [81-83].

Surprisingly, recent studies have also revealed an oncogenic potential for wild-type p110γ. It was shown that over-expression of this isoform induced oncogenic transformation in chicken embryo fibroblasts and moreover, that the lipid kinase activity of p110γ was essential for transformation [84]. However, cancer-specific mutations in p110γ have not been reported to date [84].

More recently, a study revealed a crucial role of p110γ in electrotaxis-regulated wound healing. Targeting the p110γ gene in mice led to a decrease in electrical field-induced signaling and attenuated the directed migration of healing epithelium [85]. Thus, the first gene involved in cell movement and wound healing in response to electrical signals was identified, making p110γ a very promising tool to modulate the wound healing process.

Ten years after the discovery of p110γ, studies on this key enzyme still uncover new biological functions and the initial hypothesis that targeting this enzyme could have a significant therapeutic benefit could be confirmed by studies using novel specific pharmacological inhibitors.

Phosphatidylinositol 3-Kinase p110δ Catalytic Subunit [61, 86] and Inhibitors of Phosphatidylinositol 3-Kinase p110δ [64, 87]

The class I_A PI3K catalytic subunit p110δ was identified and isolated as the last isoform within this class of PI3Ks [6]. A patent on polynucleotides encoding p110δ and recombinant p110δ polynucleotides, antibodies to p110δ and assays for identifying inhibitors of p110δ was issued in 1999 by Chantry *et al*. [61, 86]. Their invention was initially based on the identification and isolation of a novel lipid kinase closely related to the other class I PI3K isoforms. The enzyme displayed substantial sequence homology with p110β (72%), p110α (49%) and p110γ (45%) in the carboxy-terminal catalytic domain. The newly identified cDNA sequence encoding the lipid kinase was designated p110δ and classified in the class I_A of the PI3K family.

The cloning and characterization of p110δ was based on a strategy of amplification of conserved PI3K cDNA sequences. In a PCR analysis, degenerate oligonucleotide primers based on conserved sequences in the catalytic domain of known PI3Ks were used to screen a human cDNA library [6]. The identified full length human p110δ cDNA comprised an open reading frame of 3135 nucleotides, encoding a protein of approximately 114 kDa. Similarly to p110β, the catalytic domain of p110δ was found to be located in the C-terminus of the protein. The functionality of the newly identified PI3K was shown by a lipid kinase activity assay after transfection of cells with epitope-tagged p110δ. Production of PI 3-phosphates demonstrated a functional PI3K enzyme activity, which was sensitive to the PI3K inhibitor wortmannin. Similarly to the other class I_A PI3K, which are known to form heterodimers with a p85

subunit, co-immunoprecipitation studies revealed the ability of p110δ to associate with the adapter protein p85. Expression analysis in various human tissues revealed an abundant distribution of p110δ in lymphocytes and lymphoid tissue, suggesting a role in the immune system [6].

The identification of p110δ as another member of the PI3K family opened an interesting avenue for further investigations. The whole family of PI3Ks has been shown to be of high importance in the control of various cellular responses, which has made them interesting targets for research and clinical applications. The described patent includes various of these possible applications.

As a protein involved in signal transduction, the p110δ polypeptide is an interesting target for interaction studies. Its use involves the generation of fragments modulating the binding of p110δ and a binding partner, or analogs with additions, substitutions or deletions in order to increase or decrease the binding affinity of p110δ to a binding partner. Furthermore, modification of the polypeptide might facilitate its passage into the cell and help control its localization. Antibodies specifically immunoreactive with p110δ provide another useful tool to modulate the binding affinity of p110δ and can further be used for purification of the protein, its detection and quantification in biological samples. Another aspect is that the knowledge of the polypeptide provides a base for the identification of modulators that affect the binding affinity, or the functionality of p110δ, as well as the expression levels of the protein.

With respect to human diseases, the chromosomal localization of certain genes is of high interest to identify deregulated expression of their transcripts. As elevated function of PI3Ks has been shown to play a role in carcinogenesis, the polynucleotides described in the invention may be useful tools for chromosomal localization studies. Furthermore, the above described antibodies could constitute valuable diagnostic tools.

A patent on methods for inhibiting p110δ activity, and methods for treating diseases was deposited by Sadhu *et al*. in 2003 [64, 87]. The methods employ active agents that selectively inhibit p110δ, while not significantly inhibiting other PI3K isoforms. The applications of these p110δ inhibitory compounds are of high interest for the treatment of diseases such as disorders of immunity and inflammation. These inhibitors could also be used to inhibit cancer cell growth and proliferation. Furthermore, methods for the inhibition of p110δ-mediated processes *in vitro* and *in vivo* were included in the described patent.

Generic PI3K inhibitors such as LY294002 [18] or wortmannin [16, 17] have contributed a lot to the understanding of these kinases. The identification of specific functions of the different PI3K isoforms, however, requires more targeted approaches and forced the quest for substances acting on only one specific enzyme. The described invention provides the structure of several compounds displaying selective inhibitory activity towards p110δ. The compounds were selected to be at least 10-fold more selective for p110δ, when compared to other PI3K isoforms. The described compounds inhibit the biological activity of human p110δ and represent a useful tool to further characterize the functions of p110δ. Another aspect of the invention is that the compounds provide methods to selectively modulate p110δ activity, which represent useful tools for the treatment of diseases caused by p110δ dysfunction.

An embodiment for the use of the described patent includes the methods for inhibiting the growth and proliferation of cancer cells. The PI3K p110δ has been suggested to play a role in cancer development and a study has shown that p110δ enhances the radiation-induced tumor control in murine cancer models [88]. Treatment of mice with Lewis lung carcinoma or hind limb tumors with a p110δ-specific inhibitor in combination with radiation abrogated the radiation-induced phosphorylation of Akt and significantly reduced the tumor volume [88]. A recent study further underlined the potential of p110δ-specific inhibitors in cancer treatment by describing a reduction in cell proliferation of acute myeloid leukemia (AML) cells [89]. Treatment of AML cells with a p110δ-specific inhibitor decreased the constitutive phosphorylation of Akt found in AML cells and furthermore was synergistic in reducing cell viability in combination with the topoisomerase II inhibitor VP16 [89]. Another field of application results from the known role of p110δ in B cells. Studies have shown an involvement of p110δ in the recruitment and activation of certain inflammatory cells [90]. Inhibition of p110δ impaired the movement of neutrophils across inflamed venules [90]. Furthermore, specific inhibition of p110δ activity attenuated the allergic airway inflammation and hyper-responsiveness in a murine asthma model [52]. Treatment of model mice with a p110δ-specific inhibitor significantly reduced antigen-induced airway infiltration of inflammatory cells, secretion of T_H2 cytokines in the lungs, as well as vascular permeability [53].

Mutations of the *PIK3CA* Gene in Human Cancers [91], Condensed Heteroaryl Derivatives and their Use as Inhibitors [65]

The p110α isoform was described as a retroviral oncoprotein that can transform chicken embryo fibroblasts *in vivo* [92]. Its key role in tumorigenesis was further supported by accumulating evidence indicating that p110α gain-of-function by over-expression or by somatic missense mutations is common in many human cancers. The *PIK3CA* gene consists of 20 exons encoding a protein of 1068 amino acids and 124 kDa size. The 3q26 locus where it is located is amplified in several human cancers, including head and neck cancers [93], cervical cancers [94], gastric cancers [95] and lung cancers [96]. In a landmark study, Samuels *et al*. performed a large-scale sequence analysis of 8 PI3K and 8 PI3K-related genes in human cancers and discovered that *PIK3CA* was the only gene harboring somatic mutations [43]. Furthermore, they reported a surprisingly high proportion of colorectal cancers (32%), gastric cancers (25%) and glioblastomas (27%) carrying somatic mutations in *PIK3CA*. These mutations also occurred in a smaller fraction of lung cancers (4%) and breast cancers (8%). Interestingly, all mutations were apparent activating missense mutations that were found to cluster primarily in two major "hot-spots", E545 in the helical phosphatidylinositol kinase homology domain, and H1047 in the catalytic domain (exon 9 and exon 20, respectively).

Clearly, the clustering of mutations implies that *PIK3CA* sequence analysis may prove useful for diagnostic purposes, as a marker for early detection of cancers, or for monitoring tumor progression. Furthermore, these data provide a reasonable case for considering p110α as a potential target for pharmacological intervention. Subsequent studies expanded these findings and collectively found *PIK3CA* mutations in a wide range of tumors, such as glioblastoma (5% and 27%), medulloblas-toma (5%), breast cancer (18%, 25%, 27% and 40%), lung cancer (4% and 1%), gastric cancer (6.5%), ovarian cancer (6.6% and 12%), liver cancer (36%) and acute leukemia (1%) [43, 97-100].

Samuels *et al.* patented their discovery [91], which comprises methods for assessing cancer on body samples from a human suspected of having cancer based on the detection of intragenic mutation in a *PIK3CA* coding sequence. The invention also includes several methods for inhibiting progression of a tumor in a human by reducing the expression, or inhibiting the activity of p110α, a method for delivering an appropriate chemotherapeutic drug to a patient in need thereof, and a set of one or more primers for amplifying and/or sequencing *PIK3CA*.

The discovery of non-random somatic mutations in the *PIK3CA* gene in a wide range of human tumors strongly pointed to an oncogenic role for the mutated enzyme. In order to elucidate the consequences of *PIK3CA* alterations, Kang *et al.* determined the growth-regulatory and signaling properties of the three most frequently observed PI3K mutations: E542K, E545K, and H1047R. When expressed in chicken embryo fibroblasts, all three mutants induced oncogenic transformation with a high efficiency and lead to constitutive increases in the phosphorylation of Akt, p70 S6 kinase, and 4E-binding protein-1 [42]. This transforming ability correlated with elevated catalytic activity in *in vitro* kinase assays. The expression of these mutant *PIK3CA* enzymes conferred Akt activation in the absence of growth factor stimulation, promoted cell growth and invasion [42, 101]. Moreover, treatment with the PI3K inhibitor LY294002 abrogated PI3K signaling and preferentially inhibited growth of *PIK3CA* mutant cells.

This remarkable progress in our understanding of the role of the PI3K p110α in tumorigenesis allowed the development of new therapeutic strategies for the treatment of cancers harboring *PIK3CA* gene amplifications or mutations. Recently Hayakawa *et al.* carried out a high-throughput screen to identify novel p110α inhibitors and 4-Morpholino-2-phenylquinazolin was discovered as having p110α-inhibitory activity [102]. A series of derivatives were synthesized from this lead compound. Introduction of a 3-hydroxy group on the phenyl group and replacement of the quinazoline ring with a thieno [3,2-*d*]pyrimidine ring resulted in 3-(4-Morpholinothieno[3,2-*d*]pyrimidil-2-yl) phenol hydrochloride, also designated YM024. This compound is a highly selective inhibitor of p110α, when compared to other kinases and PI3K isoforms, with an IC_{50} value of 2.0 nM, making it the first example of a p110α–selective PI3K inhibitor. These series of compounds were patented in 2003 by the same group [65], claiming a pharmaceutical composition for a PI3K inhibitor comprising a fused heteroaryl derivative or a salt thereof and a pharmaceutical acceptable carrier. Moreover, patents exist for their use in the manufacturing of medicaments for use in the treatment of a disorder in which PI3K plays a role, and for use in the treatment of cancer. They also describe a method to treat disorders which are associated with PI3K and to treat cancer by administering an effective amount of the fused heteroaryl derivative to a patient [65]. According to the invention, the pharmacological effects of the compounds have been verified by several pharmacological tests. Compounds of the present invention exhibited an excellent p110α-inhibitory activity, with IC_{50} values below 1 μM, whereas others were confirmed to have inhibitory activities against other PI3K isoforms, such as PI3KC2β. Several compounds exhibited an excellent growth-inhibitory activity *in vitro* against colon cancer, melanoma, lung cancer, glioma, ovary cancer, prostate cancer and pancreas cancer. Furthermore, inhibition of cancer cell growth was assessed *in vivo*. HelaS3 cells, a human cervix cancer cell line, were inoculated into the flank of female BAlb/c nude mice by subcutaneous injection. When the tumor reached 100-200 mm^3 in volume compounds were intra-peritoneally adminis-trated once a day for 2 weeks and tumor volume after treatment was determined. The test compounds exhibited superior anti-tumor activities as compared with the control group.

YM024 has also already proven to be a valuable research tool for the investigation of isoform-specific p110α functions, not only in cancer, but also in other PI3K-mediated processes, such as inflammation [103].

PIK3CA represents one of the most frequently mutated oncogenes identified to date and one of the few established human oncogenes that is commonly activated through either gene amplification or point mutations. Notably, no mutations in the other PI3K catalytic subunits have been reported in human cancer until the present date, suggesting that the PI3K p110α harbors a selective oncogenic potential. The oncogenic transforming activity of the mutant p110α proteins makes them promising targets for small molecule inhibitors that could be further developed into effective and highly specific anticancer drugs.

Methods for Modulating T cell Responses by Manipulating Intracellular Signal Transduction [104]

T lymphocytes are cells involved in the adaptive immune response (secondary response), which in case of infection are able to provide a more specific response than the innate immune system (primary response). T cell precursors, produced by the bone marrow, migrate to the thymus, where they undergo complete differentiation and selection. In the thymus, T cells come in contact with self MHC-peptide complexes expressed on antigen-presenting cells (APCs) to which the T cells should not react. T cells that show the appropriate affinity for the self MHC-peptide complexes are allowed to migrate to the secondary lymphoid organs (spleen and lymph nodes), while the others are eliminated [105]. Immature T lymphocytes express both the CD4 and CD8 co-receptor molecules on their surface, but after the selection and maturation process they express only one of them. While $CD4^+$ cells recognize non-self antigen presented on the surface of APCs by MHC class II molecules and become

cytokine-secreting cells, $CD8^+$ cells recognize non-self antigen presented by MHC class I and become cytotoxic effector cells. APCs include cells such as dendritic cells, Langerhans cells and natural killer cells (NK).

In the secondary lymphoid organs, the lymphocytes are in a state of anergy, until they encounter APC cells, which present a non-self peptide in complex with MHC class I or II molecules on their cell surface (either from a pathogen or a cancer cell) [106]. Mature T cells recognize MHC-non self peptide complexes via their transmembrane T cell receptor (TCR). However, this interaction is not sufficient to generate a proper immune response against the antigen. For complete T cell activation, an interaction with co-stimulatory ligands, such as CD80 and CD86 expressed on the surface of the APCs is necessary. These co-stimulatory ligands are able to bind to the CD28 receptor on the surface of T cells. The signals from this co-stimulatory receptor, in combination with those from the TCR, activate the cell and initiate gene transcription, cell growth and mitosis. One of the most important effects of the co-engagement of TCR and CD28 receptors is the production of IL-2, a cytokine that provides strong proliferative and survival signals.

Several studies have provided clear evidence for a rapid recruitment and substantial activation of PI3K in T cells, which drives the production of D-3 phosphoinositides as signal mediators. Activation of T cells induces a time-dependent elevation of the described second messengers, which remain high until nine hours after activation and then decrease back to normal levels. Subcellular localization studies revealed a major presence of these phosphoinositides in the contact area between the T cell and the APC [107].

Further evidence for an involvement of PI3Ks in proliferation signals in T cells was provided by the fact that mouse T lymphocytes stimulated with an anti-CD3 antibody showed a decrease in their proliferation rate in the presence of the PI3K inhibitor LY294002. In T cells from mice carrying a mutation in the PI3K catalytic subunit p110δ ($p110\delta^{D910A/D910A}$), a reduction in Akt/PKB phosphorylation, as well as in $CD4^+$ T cell proliferation in response to CD3 stimulation was observed [28, 108]. The proliferation capacity of $CD4^+$ cells was restored upon co-stimulation with CD28. These results supported other studies describing an important role for PI3K in TCR signalling independently of CD28, even though the molecular mechanism by which the TCR is coupled to PI3K is still unclear [109, 110]. Similar experiments performed in p110δ knock-out mice yielded less clear results, which might be caused by a greater capacity of p110α and p110β to compensate for the absence of p110δ expression [29].

Interestingly, it was shown that CD28 can activate PI3K independently from the TCR and that CD28 contains a Tyr-X-X-Met motif in its cytoplasmatic domain that can bind the SH2 domain of p85 [107, 111, 112]. However, the exact role played by CD28 and its cytoplasmatic domain in the activation of PI3K is still not totally understood. CD28-deficient mice expressing a CD28-Tyr170Phe mutant (Phenylalanine to Tyrosine substitution in the PI3K binding motif of CD28), were still able to co-stimulate T cell proliferation and IL-2 production independently of CD28 association with PI3K [113]. Instead, the response which was totally abrogated in such mutants was the capacity of CD28 to activate Bcl-X_L and promote T cell survival [113, 114]. On the other hand, the capacity of CD28-deficient mice to proliferate and produce IL-2 was also restored by expression of an activated mutant of Akt/PKB, which seems to be in conflict with the previous observation [115]. Despite the fact that the ability of CD28 to recruit PI3K might be dispensable for T cell proliferation and IL-2 production, it is still possible that CD28 can influence the capacity of the TCR to couple to PI3K and activate Akt/PKB independently of the CD28-Tyr170. Furthermore, the cytoplasmatic domain of CD28 contains two Proline-rich regions (PYAP) that are able to facilitate the recruitment of p85 through its SH3 domain [116, 117]. Taken together, it appears that the activation of PI3K due to CD28 triggering can be *direct*, through the involvement of the YXXM region (Fig. (**4**)), or *indirect*, through the involvement of the PYAP region and consequent involvement of proteins such as Lck and ZAP-70.

A patent on methods for modulating T cell responses by manipulating intracellular signals associated with T cell co-stimulation was deposited by June in 2004 [104]. The invention focuses on the inhibition or the activation of the production of D-3 poly-phosphoinositides in T cells, which can be achieved by manipulating PI3K signaling, or other intracellular signals associated with co-stimulation. The patent describes useful tools for clinical applications, including situations where it is desirable to inhibit an immune response to an antigen(s), e.g. organ or bone marrow transplantation and autoimmune diseases.

The evidence that CD28 activation plays a crucial role in T cell activation and that PI3K plays a central role in the CD28 cascade focused the attention of research on the possibility of suppressing the immune system by modulating PI3Ks and the signal cascade controlling its activation. The best described PI3K inhibitors are wortmannin [16, 17] and the quercetin derivative LY294002 [18]. However, their lack of isoform specificity, as well as their high overall toxicity limits their utility with respect to clinical applications. In view of the limitations of generic pharmacological inhibitors, there is a need to find other agents that can be used alone, or in combination with these PI3K inhibitors. A main result of combining different drugs that act by different mechanisms is that a very powerful immunosuppressive effect can be achieved. This makes it possible to administer low doses of each single drug, which reduces drug-related toxicity. The use of compounds displaying higher specificities, such as inhibitors of the PI3K isoforms p110δ or p110γ would also have great advantages and are described in this review [60, 64]. An important benefit of specifically suppressing the immune system is that the body is still able to maintain a sufficient level of host defense to protect itself against infections and malignancies.

A new strategy directly connected to the suppression of CD28 activation derives from the evidence that the cytotoxic T lymphocyte antigen 4 (CTLA4) receptor binds to the same APC ligand (CD80-CD86) as CD28 [118, 119]. The interaction results in an inhibition of IL-2 production, cell-cycle progression and of the activation of TCR-induced cyclins through blockage of JNK, Erk and NF-κB activation

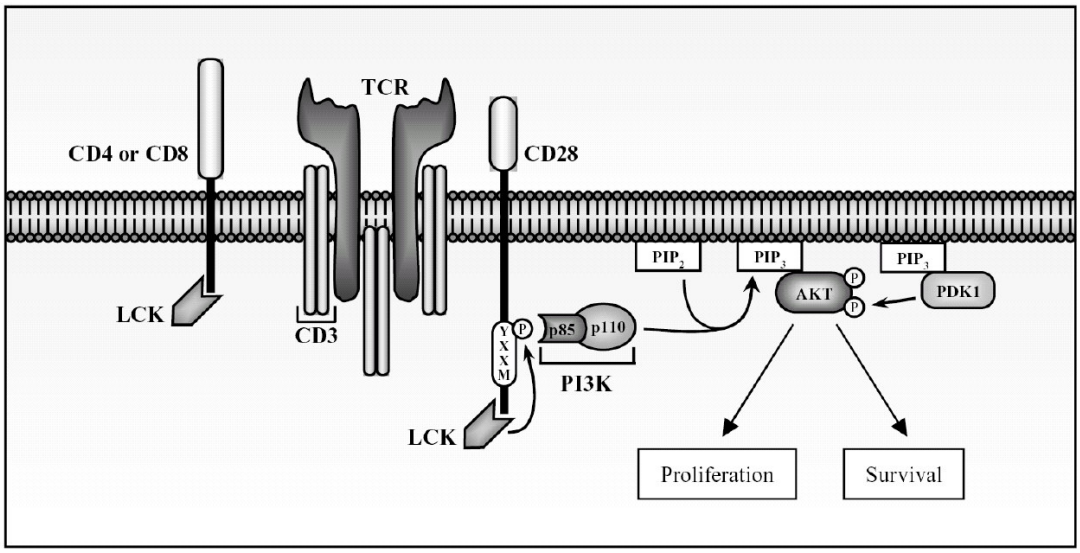


Fig. (4). Involvement of PI3K in T cell receptor (TCR) signaling. The cytoplasmatic tail of the CD28 co-receptor contains a YXXM motif, to which the PI3K regulatory subunit p85 can bind upon tyrosine phosphorylation. While PI3Ks have the potential to regulate different signaling pathways in T cells, the exact role they play is not yet clear.

[120-122]. It would thus be possible to block CD28 engagement and induce CTLA4 triggering by treating patients with a B7-specific fusion protein CTLA4-Ig. The use and toxicity of this molecule is, however, a controversial issue and thus it was used in different approaches depending on the disease. In human bone marrow transplantation, CTLA4-Ig has an *in vitro* use to prevent graft-versus-host disease (GVHD). Its use *in vivo* is common in case of allograft rejection for kidney and heart, as well as for the treatment of psoriasis [123, 124]. The effect of CTLA4-Ig might further be improved by a combined use with a CD40L-specific monoclonal antibody that induces CTLA4 over-expression [125, 126].

Several other drugs/agents are already under study, some of which directly target the different steps of the extracellular signal-regulated kinase (Erk) cascade, Jun N-terminal kinase (JNK), or p38 MAPK and are commonly used *in vitro* for interference with cytokine production. Current immune suppressive therapies which are used mainly in organ and bone marrow transplantation and occasionally in rheumatoid arthritis are Cyclosporine A (a fungal metabolite extracted from *Tolypocladium inflatum gams)* and Tacrolimus (derived from the soil fungus *Streptomyces tsukubaensis)*. These two molecules are able to block the translocation of the nuclear factor of activated T cells (NFAT) to the nucleus. In this way these compounds block IL-2 production and cell proliferation, which are the first steps in T cell activation [127-129].

All these different kinds of inhibitory approaches can be combined with the PI3K inhibitors described before. In this way, the problems resulting from the use of wortmannin or quercetin-derived inhibitors alone could potentially be overcome.

There are two main types of applications for inhibitors of T cell activation, which are *in vivo* and *in vitro*. In the first case, the PI3K inhibitor, together with the other agents chosen (especially in the case of antibodies), are directly injected into the patient. For example, in case of organ transplantation, the agents are injected together with the transplant and maintained by follow-up injections. However, this approach is not always successful, depending on the toxicity of the agents and possible unspecific collateral effects on other cells. In the case of transplantation, an *in vitro* approach is therefore often preferred. In this case, a state of anergy has to be induced in order to avoid the GVHD which happens in allogeneic transplantation. This approach is based on cells from a donor cultured together with a CD3-triggering agent and a CD28 inhibitor. At the time when the T cells are totally anergic, they can be transplanted into the patient together with the cells needed.

Most of what has been described above relates to the capacity to trigger CD28 and its related proteins in order to induce T cells anergy. However, these approaches can also be used for immune system stimulation, which is especially beneficial to induce tumor rejection. The basis of this approach is the possibility to produce a stronger co-stimulation for specific T cells that have already received a primary activation signal through TCR triggering. The most important application of PI3K activation/co-stimulation is in cancer immune therapy. In general, chemoresistant cancer cells that express specific antigens on their surface are able to induce T cell activation. However, this activation is often too slow and too low for a complete elimination of the cancer cells [130]. An approach to boost the signal for the immune system is the implementation of a co-stimulation, independently of the presence of APCs. Some drugs already commonly used in the clinic are thalidomide analogs such as

ImiDs and SelCIDs. Their role in co-stimulation of T cells has already been demonstrated [131]. As already discussed in the case of immune system tolerance, the use of these compounds can be planned *in vitro* or *in vivo*.

In vitro cancer immunotherapy can be divided into passive and adoptive methods, depending on the characteristics of the lymphocytes generated and injected into the patient. In the case of the adaptive immunotherapy, lymphocytes are cultured in the presence of a specific antigen and a CD28 stimulator (or agents that induce co-stimulation). In this case, the T lymphocytes generated and re-injected into the patient are able to directly attack the tumor cells [132, 133]. In contrast, lymphocytes generated for the active immunotherapy are not themselves specific for the antigen, but are able to stimulate the patient's immune system in order to respond better and faster to the antigen. The latter method can also be used in patients with a temporary immune deficiency in order to generate a temporary immune protection against common pathogens [134].

In vivo immunotherapy relates to the possibility of inducing a stronger and faster immunity without any cellular vaccination. Patients are treated with direct injections of agents which provide a stronger and longer co-stimulatory signal by acting directly on specific T cells that have already received a signal through *in vivo* TCR engagement.

The growing understanding of PI3Ks and their possible role(s) in the immune response opened an interesting field of research into the identification of better and safer immunosuppressive or immunostimulatory drugs. However, an improvement in short and long term outcome of the desired immune response is still needed. Multiple potential PI3K modulators have recently been described and screening assays will hopefully reveal their potential uses in immunotherapy. However, *ex vivo* studies still have to be performed for a better understanding of the capacity of drugs targeting PI3K activity to modulate immune responses. Systematic studies in animals and human patients will then be required to bridge the transition in drug development from target enzymes to the modulation of lymphocyte function.

CURRENT & FUTURE DEVELOPMENTS

The patents described in the present review have a wide range of potential applications in medical research, including the development of novel anti-cancer drugs and anti-inflammatory agents. This is the consequence of the various biological functions of distinct PI3K isoforms, which have been characterized in the past years. Gene targeting and transgenic mouse models have been instrumental in describing the distinct roles of the p110γ and p110δ isoforms in the immune system. These studies have been confirmed by the use of isoform-specific pharmacological inhibitors, which are now further being developed as novel drugs for diseases such as rheumatoid arthritis and allergies. In the case of p110α, the discovery of somatic mutations in the *PIK3CA* gene in a substantial number of different human cancers has made this PI3K isoform a validated target for the development of anti-cancer agents. However, in view of the complexity of the signaling networks involving PI3K isoforms [24, 32, 135], it remains unclear how soon drugs targeting these enzymes will successfully achieve the transition from the bench to the bedside.

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ARTICLES and REVIEWS

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The catalytic class IA PI3K isoforms play divergent roles in breast cancer cell migration.
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Targeting PI3KC2β impairs proliferation and survival in acute leukemia, brain tumours and neuroendocrine tumours
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